Studies of GH Receptor Signalling and Antagonism in the setting of Growth Hormone Deficiency and Acromegaly

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Abstract

Conditions of GH excess and deficiency cause significant morbidity and mortality. Treatments for both situations have evolved considerably in recent years, but heterogeneity in therapeutic responses remains poorly understood. An improved understanding of the role of the growth hormone receptor' (GHR) has the potential to advance future clinical management. Deletion of exon 3 in the GH receptor (d3-GHR) has been linked to enhanced rhGH responsiveness in children; the effect in adults with GH deficiency and acromegaly in adults is less well understood.

Pegvisomant, a GHR antagonist is a highly effective treatment for acromegaly but monitoring of treatment is limited by the potential imprecision of IGF-I as the sole marker of response.

The aim of this work was two-fold; to investigate the effect of the d3-GHR in determining an individual's response to GH in GH deficiency and acromegaly and to investigate the effect of supraphysiological doses of pegvisomant on IGF-I and the physiological markers of GH activity in patients with acromegaly.

194 GHD patients and 79 acromegaly patients were genotyped for d3-GHR and results correlated with clinical and biochemical response to GH. Homozygosity for d3-GHR confers a marginal increase in GH responsiveness in GH deficiency and acromegaly but without significant clinical effects. Both d3 alleles are required to achieve this response; given that only 10% of the population are d3 homozygotes, d3-GHR does not explain heterogeneity in GH responsiveness.

Investigation of supra-physiological doses of pegvisomant revealed unexpected and previously unpublished findings; despite two to four fold increases in dose, six of the nine patients failed to achieve target subnormal IGF-I levels.

The absence of a significant role for d3-GHR in determining GH response and the unexpected difficulty in causing GH deficiency with high dose GH receptor antagonism highlights the need for further study of the GHR in determining an individual's response to GH.

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Statement of Originality

I, Veronica Moyes, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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Dr Veronica Moyes.....

Details of collaboration and publications:

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All work was performed by myself; other authors provided support in a supervisory role, provided patients for the study or provided logistical support.

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Abbreviations

AcroQol	Acromegaly Quality of Life assessment
AGHDA	Adult Growth Hormone Deficiency assessment
DA	Dopamine agonist
E2	Oestrogen
EBRT	External Beam Radiotherapy
GH	Growth Hormone
GHBP	Growth hormone binding protein
GHD	Growth Hormone Deficiency
GHR	Growth Hormone Receptor
d3-GHR	GHR with exon 3 deletion
fl/fl	Homozygous wild-type isoform for growth hormone receptor
fl/d3	Heterozygous isoform for exon 3 deletion of the growth hormone receptor
d3/d3	Homozygous isoform for exon 3 deletion of the growth hormone receptor
IGF-I	Insulin like growth factor I
IGF-I%ULN	IGF-I % upper limit of normal reference range
ISS	Idiopathic short stature
PCR	Polymerase Chain Reaction
rhGH	Recombinant Human Growth Hormone
SST	Somatostatin analogue
SGA	Small for gestational Age

Chapter 1

Introduction

Although the original study of endocrinology started in 200BC in China, endocrinology is a relatively young medical specialty with advances in our understanding occurring largely over the past century. Historically, the pituitary was believed to be responsible for the regulation of the secretion of phlegm until the 17th century when Richard Lower speculated that substances passed from the brain to the pituitary and thence to the blood. The term “hormone” only came into use 100 years ago following Starling’s work, demonstrating the presence of a substance obtained from pancreas which produced biological effects in the absence of innervation.

The development of modern clinical neuroendocrinology dates back to the early 20th century as a result of the observations and experiments of Harvey Cushing, a Baltimore and Boston neurosurgeon. He performed hypophysectomies on dogs, establishing the true function of the pituitary by recording the consequences of this intervention. He coined the terms hyper- and hypopituitarism to describe the consequences of pathological pituitary function (Cushing 1910). In 1912 he proposed the existence of a “hormone of growth” the primary action of which was to control and promote skeletal growth. Furthermore he also identified the causative link between pituitary tumours and conditions of pituitary hyperfunction, namely acromegaly and ACTH excess, now commonly referred to as Cushing’s disease.

The identification of a somatic growth enhancing factor arising from the pituitary occurred in 1921 following the intraperitoneal administration of crushed bovine pituitary tissue into rats (Evans and Long Anatomy Record 21, 62-63). Human Growth hormone (GH) was first isolated from human pituitaries obtained at autopsy in 1951 (Raben 1962) and since then has been used in the treatment of conditions of GH deficiency and short stature, originally as a cadaveric extract and more recently in its recombinant form.

The last two decades have seen an exponential increase in our appreciation and understanding of the effects of GH on the body. The translation of our understanding into clinical practice has resulted in the development of effective treatments for conditions of GH deficiency (GHD) and GH excess.

In spite of our improved understanding, important and obvious questions remain unanswered. We cannot yet explain why there is such variability in an individual's response to treatment. For example doses of recombinant GH (rhGH) used in the treatment of adult GHD can vary up to four-fold. Questions also persist with regards to conditions of GH excess; there is great variability in disease severity but also a significant discrepancy in the biochemical presentation of the disease with variability in the levels of IGF-I generated for a given level of GH. Another question is how we might optimise our treatment regimens for patients with difficult to control GH excess; a significant proportion of patients remain uncured despite surgery and radiotherapy and require ongoing treatment. The development of newer agents such as the GH receptor antagonist pegvisomant has led to a significant improvement in symptom and biochemical control, but there remain unanswered questions regarding its usage; how can we refine our monitoring of treatment when serum IGF-I remains our only guide, and why is there such variability in dose requirements.

This thesis aims to investigate the factors potentially responsible for governing variability in clinical response to treatment and to investigate the optimal method of monitoring response to treatment, using models of GH deficiency and GH excess as examples. By improving our understanding of these underlying mechanisms, this will potentially allow the future optimisation and tailoring of treatment to an individual's needs.

1.1 Growth Hormone: Production and Regulation

Growth hormone (GH) is a protein-based peptide hormone responsible for stimulating growth, cell reproduction and regeneration. It is structurally and functionally similar to prolactin and placental lactogens (Nicoll 1982); (Goffin and Kelly 1996). The genes that encode GH and other members of this hormone family are believed to have evolved over the last 350 million years via the duplication of a common ancestral gene (Miller and Eberhardt 1983).

The GH gene is located on the long arm of chromosome 17 and is approximately 3kb long, consisting of 5 exons and 4 introns. This encodes a 217 amino acid precursor protein (Miller and Eberhardt 1983) which generates a single chain polypeptide containing 191 amino acids following proteolytic cleavage of the aminoterminal signal peptide. The majority of GH is the 22kDa isoform, however as a result of alternate splicing of the GH precursor mRNA up to 25% consists of 20kDa and other isoforms (Baumann 1991).

GH is synthesised, stored, and secreted by the somatotroph cells within the lateral wings of the anterior pituitary gland. The morphological characteristics and number of these cells are remarkably constant throughout life; it is the level of secretion that varies according to physiological requirements. GH is secreted in a pulsatile fashion, pulses occurring every 3-4 hours with maximal secretion occurring in the second half of the night. Secretion is predominantly regulated by two hypothalamic hormones; Growth Hormone Releasing Hormone (GHRH) and somatostatin (SST).

The importance of the hypothalamus in regulating GH secretion was recognised by Reichlin's experiments in 1961 (Reichlin 1961) demonstrating a reduction of GH content of the pituitary and a reduction in growth in rats with lesions of the ventral hypothalamus. Furthermore, Deuben and Meites demonstrated that rat

hypothalamic extracts could stimulate GH release from the rat pituitary in vitro in 1964 (Deuben and Meites 1964). GHRH has since been isolated and confirmed as the main stimulant of GH synthesis. It is released from neurosecretory nerve terminals of these arcuate neurons, and is carried by the hypothalamo-hypophyseal portal system to the anterior pituitary gland. It then stimulates the growth hormone releasing hormone receptor (GHRHR), causing an increase in cytosolic calcium which leads to GH release.

The primary function of somatostatin (SST) is to inhibit GH release but not its synthesis. Its existence was first postulated by Krulich et al in 1968 using hypothalamic extracts that were able to inhibit the GH secretion (Krulich, Dhariwal et al. 1968). SST was first isolated in 1973 by Brazeau (Brazeau, Vale et al. 1973) with the gene sequence characterised in 1984 (Shen and Rutter 1984). It binds to a family of specific receptors; all subtypes are expressed in pituitary tumours and in normal foetal pituitary tissue (Miller, Alexander et al. 1995).

Other GH stimulating and inhibitory factors exist including physiological factors such as stress, hypoglycaemia and ingestion of protein induce GH release and hyperglycaemia and raised free fatty acids inhibits GH secretion. Over recent years a class of molecules known as GH releasing peptides including ghrelin, have been found to be a potent stimulator of GH secretion increasing the release of GHRH and inhibiting SST action. The identification of ghrelin which is secreted and acts via the the GH secretagogue receptor in the stomach, suggests that GH release can be regulated in the periphery rather than purely by the hypothalamus (Adams, Huang et al. 1998) (Howard, Feighner et al. 1996).

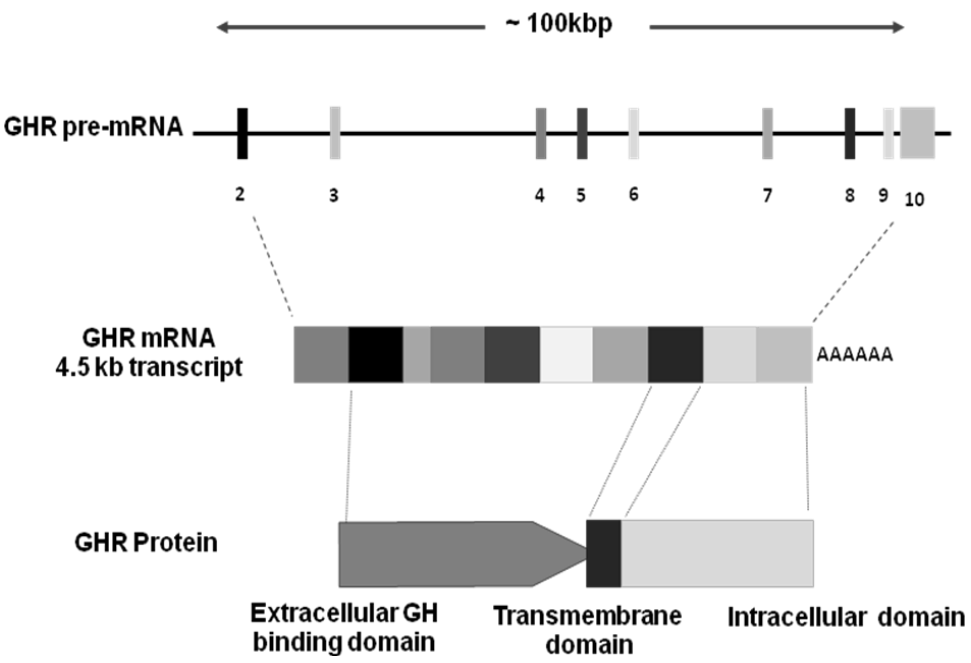
1.2 GHR

The GHR is a member of the class I cytokine receptor superfamily which includes receptors for prolactin, erythropoietin and the interleukins. The human GHR gene is located on the short arm of chromosome 5 in the region p13.1-p12 and contains 9 coding exons; the 5' untranslated region of this gene contains multiple exons that alternatively serve as exon 1, exon 2 codes for the signal peptide, exons 3–7 encode the extracellular domain, exon 8 codes the transmembrane domain and exons 9 and 10 code the cytoplasmic domain (Godowski, Leung et al. 1989) (Figure 1.1).

The GHRs comprise a single polypeptide chain 614–626 amino acids in length with a predicted molecular mass of approximately 70 kDa. They were first identified in hepatic tissue but are located in a variety of tissues such as bone, heart, kidney, muscle and adipose tissue, and more recently in immune tissue such as the spleen and thymus (Kelly et al 1991; (Mertani, Delehaye-Zervas et al. 1995); (Ohlsson, Nilsson et al. 1993); (Nyberg and Burman 1996); (Hill, Riley et al. 1992); (Hull, Thiagarajah et al. 1996); (Rapaport, Sills et al. 1995).

Figure 1.1 The GH Receptor and mRNA transcript

This diagram is a representation of the GHR gene which is located on the short arm of chromosome 5 (p13.1-p12) and contains nine coding exons, with exons 3-7 encoding the extracellular ligand binding domain and the mRNA transcript.



1.2.1 Activation of the GHR

A molecule of GH binds to two GH receptors, a high affinity site on one GHR monomer causing rotation of the intracellular domains to allow the lower affinity binding of this molecule to a second GHR monomer. This increases affinity of both receptors for JAK2 tyrosine kinase, which phosphorylates the GHR leading, in turn, to phosphorylation of a number of intracellular proteins including the signal transducers and activators of transcription or STATs. These form homo or heterodimers, enter the nucleus and regulate GH specific gene transcription. This allows the expression of genes involved in anabolic processes including protein synthesis, lipid degradation, immune function, muscle mass, and bone turnover (Wang, Moller et al. 1993); (Argetsinger, Hsu et al. 1995); (Frank, Gilliland et al. 1994); (Wang, Darus et al. 1996); (Wang, Xu et al. 1994, Wang, Souza et al. 1995). Intracellular growth hormone signalling is suppressed by several proteins, especially the suppressors of cytokine signalling (SOCS) (Krebs, Uren et al. 2002).

1.2.2 GHBP

Under normal physiological conditions half of circulating GH is bound to a specific binding protein GHBP (Baumann, Amburn et al. 1988, Baumann, Vance et al. 1990). This comprises the cleaved extracellular portion of the GHR in the peripheral circulation. This serves to act as buffer in the circulation to smooth out oscillations in GH levels resulting from pulsatile GH secretion by prolonging half-life and reducing renal clearance.

1.2.3 Biological effects of GH

The somatomedin hypothesis was originally proposed in 1957 by Salmon and Daughaday; this states that the observed effects of GH are mediated by the activation of IGF-I (Salmon and Daughaday 1957). The dual effector of GH action theory was postulated in 1985; this states that the action of GH is to induce differentiation of target cells directly but also to induce secretion of IGF-I (Green, Morikawa et al. 1985). It remains unclear which of the effects of GH are directly mediated and which are IGF-I mediated.

GH is responsible for promoting linear growth in childhood. It acts on the germinal zone of the growth plate to stimulate proliferation and differentiation of prechondrocytes and also promotes protein synthesis and mineral retention in bone and calcium absorption from the gut. It also has a complex range of physiological effects that continue after childhood which have become more clinically apparent over recent years. GH promotes protein anabolism and the diversion of amino acids from oxidative to protein synthetic pathways (Griffin and Miller 1974), (Fain, Kovacev et al. 1965). Lipolysis occurs with an increase in fat oxidation with consequent reduction in subcutaneous fat (Clemmons, Snyder et al. 1987). GH also reduces insulin sensitivity; excessive levels can therefore induce type 2 diabetes mellitus.

1.2.4 IGF-I

IGF-I is a mediator of the metabolic actions of growth hormone. It is synthesised in the liver and also in the periphery. It is a 70 amino acid peptide with a molecular weight of 7.6kDa and has a high degree of sequence homology with human proinsulin (Daughaday, Hall et al. 1972), (Rinderknecht and Humbel 1978).

Production is stimulated by GH and a variety of factors may inhibit its production such as malnutrition and GH insensitivity. Approximately 98% of IGF-I is bound to one of the six binding proteins in the circulation. The majority of IGF-I is bound to IGFBP-3 and an acid labile subunit (ALS) forming a 150 KDa complex; this is unable to pass through endothelium and acts as an intravascular reservoir of inactive IGF-I. The half-life of IGF-I in the complex with IGFBP-3 and ALS is 12-15 hours compared with 10-12 minutes for free IGF-I (Jones and Clemmons 1995). IGF-I acts via the IGF-I receptor (IGF-IR), present on a range of cells throughout the body. Binding of the IGF-IR initiates intracellular signalling such as the AKT signalling pathway which is a stimulator of cell growth and proliferation and inhibits apoptosis. IGF-I also binds to the insulin receptor albeit at a much lower affinity; activating the insulin receptor at 0.1x the potency of insulin.

Although hepatic-derived IGF-I acting via its endocrine effects is responsible for the majority of the actions of GH, it has been clearly demonstrated that local production of IGF-I acting locally in a paracrine or autocrine manner, has important functions, in particular stimulation of cell proliferation and prevention of apoptosis. Gene knockout experiments demonstrated that animals with selective hepatic IGF-I loss have a normal phenotype and growth, despite marked reduction in serum IGF-I levels (Butler and Le Roith 2001).

Although its primary role is in the promotion of somatic growth, more recently IGF-I has been confirmed to have a diverse range of effects including the regulation of neural development; this has led to the investigation of potential therapeutic roles such as the treatment of neurological conditions such as amyotrophic lateral sclerosis.

IGFBP3

Insulin like growth factor binding protein 3 (IGFBP3) is a 264 amino acid peptide produced by the liver. It is the most abundant of a group of IGFBPs that transport and control bioavailability and half-life of insulin like growth factors (IGF) and in particular IGF-I. The half-life of unbound IGFI is 10 minutes whilst the complex of IGF-I/IGFBP3 is cleared more slowly with a half-life of 12 hours. As such they provide more stable levels compared to the pulsatile nature of GH secretion. Use of IGFBP3 clinically is limited by variability in assay quality particularly as heterophilic antibodies can interfere with the assay.

1.3 Conditions of Abnormal GH function

Tales of giants and dwarfs are often recounted in the literature of many cultures. There is speculation that Goliath, the giant from the biblical story, suffered from a pituitary tumour causing excessive GH secretion and possibly also causing a visual field defect which could have prevented him from seeing the stone from David's slingshot that caused his death.

In the present time, the clinical conditions commonly seen relating to GH are those of under and overproduction; growth hormone deficiency and acromegaly.

1.3.1 Growth Hormone Deficiency

Growth hormone deficiency (GHD) arises as a result of the lack of secretion of GH. A variety of aetiological factors may be responsible including pituitary tumours, pituitary surgery, congenital GH deficiency and CNS irradiation The physical

consequences of GHD vary according to the context in which it develops; children experience stunted growth whereas in adulthood, after fusion of epiphyseal plates, the main consequences of GHD are more subtle physical and metabolic effects.

GHD in childhood

Although there is an extensive list of potential causes of childhood GHD, the majority of cases are idiopathic, although there is an association with perinatal trauma. The prevalence is reported to be approximately 1:3500 children and it may present as an isolated or multiple pituitary hormone deficiency, most commonly associated with TSH deficiency. In isolated GHD, impairment of linear growth tends to occur in the first 2 years of life; this is usually preceded by normal birth weight and length. Early childhood may be associated with the development of a classical phenotype of growth failure, protrusion of the frontal bones, poor development of the bridge of the nose and delayed closure of the anterior fontanelle. Stimulation tests are required to confirm the diagnosis; clonidine or glucagon is more commonly used in the paediatric population rather than insulin due to the risk of hypoglycaemia. Genetic testing of the GH and other candidate genes should also be considered in the assessment of these patients. The main therapeutic aim of GH treatment in children with GHD is to normalise height during childhood, aiming for normal adult height.

Treatment of GHD in childhood

Recombinant human GH is used for the following conditions in childhood:

- Growth hormone deficiency

- Turner syndrome
- Prader–Willi syndrome
- chronic renal insufficiency
- born small for gestational age with subsequent growth failure at 4 years of age or later (SGA defined as either height at birth 2 standard deviations or more below the population average, weight at birth 2 standard deviations or more below the population average or weight at birth below the 10th percentile).
- short stature homeobox-containing gene (*SHOX*) deficiency.

According to NICE guidance, rhGH treatment should be discontinued in the following situations:

- Growth velocity increases less than 50% from baseline in the first year of treatment
- Final height is approached and growth velocity is less than 2 cm total growth in 1 year
- Insurmountable problems with adherence
- Final height is attained

Doses vary according to underlying condition; for GHD children receive 23–39 microgram/kg per day.

GHD in adulthood

In adulthood, GH deficiency most commonly occurs as a result of pituitary tumours and/ or treatment of such tumours. Rather than the obvious and easily measurable effect on linear growth, adult GHD presents itself in a more subtle, insidious manner with a range of symptoms such as reduced energy, lack of motivation and a greater

sense of social isolation. There are also clearly defined physical and in particular metabolic effects of GHD including increased central visceral adiposity, reduced lean body mass, reduced exercise capacity, diminished bone mass and altered lipoprotein metabolism resulting in raised LDL, triglycerides and low HDL levels. There is an increase in cardiovascular morbidity and mortality in GHD patients, resulting from the changes in body composition and metabolism.

The extent of the effects of GHD have only been fully appreciated over recent years and use of recombinant GH still varies worldwide; this is due to variability in clinical practice, availability of rhGH and in particular the financial aspect of such treatment. In the UK the National Institute for Health and Clinical Excellence (NICE) created guidelines for the use of rhGH in adults with severe GHD. The following three criteria must be met to qualify for treatment:

- Severe GH deficiency, defined as a peak GH response of less than 9 mU/litre (3 ng/ml) during an insulin tolerance test or a cross-validated GH threshold in an equivalent test.
- A perceived impairment of quality of life (QoL), as demonstrated by a reported score of at least 11 in the disease-specific 'Quality of life assessment of growth hormone deficiency in adults' (QoL-AGHDA) questionnaire.
- They are already receiving treatment for any other pituitary hormone deficiencies as required.

Treatment of GHD in adulthood

Standardised protocols exist to guide rhGH dosing using serum IGF-I levels as a guide to rhGH dose titration, aiming to maximise clinical benefit of treatment but minimise the potential risks of prolonged exposure to excess GH. GH replacement is monitored according to a combination of clinical response (well-being, body composition) and measurement of insulin-like growth factor I (IGF-I), aiming to avoid levels of this GH-dependent peptide above the upper limit of the age-adjusted normal range.

Using such protocols for dose titration, it is apparent that individual requirements for GH vary considerably between patients. For example, in the original St Bartholomew's report of 50 consecutive patients treated with an identical protocol of dose adjustment, with a target IGF-I between the median and upper end of the age-related reference range, median (range) dose requirements for males and females respectively were 0.8 (0.4-1.6) and 1.2 (0.8-2) (Drake, Coyte et al. 1998). Although oestrogen, which attenuates IGF-I production, may partially explain the variability between genders, this does not explain why dose requirements vary 4- and 2.5-fold in male and female groups respectively. Pituitary irradiation is also associated with a low- normal serum IGF-I from retained low-amplitude GH secretion (Toogood, Nass et al. 1997) however GH/IGF-I discordance is evident in non-irradiated patients. A greater understanding of the mechanisms underlying the variability in GH response would allow a more accurate, individual titration of rhGH replacement.

1.3.2 Growth Hormone Excess: Acromegaly

The first medical description of GH excess was in 1567 by Johannes Wierus in France. In his first case collection *Medicarum observationum raraum* he wrote about a female giant who made her fortune travelling and charging an entry fee. Over 25 years, having grown proportionally and uniformly and with the development of

secondary amenorrhoea at 14, she was slow moving and had coarse facial features.

However the first detailed description along with the naming of the condition of acromegaly, using the Greek words akron - extremities and megas – large, was provided by the French neurologist Pierre Marie in 1886. Enlargement of the pituitary was noted in his original description but was thought to be a secondary phenomenon rather than the cause. The link between GH excess and pituitary tumours was first made during autopsies in 1892 (Massalongo R, 1892).

Acromegaly is a rare condition affecting 60 people per million. It is most commonly due to GH hypersecretion from a pituitary adenoma but can rarely be due to excessive GHRH secretion from the hypothalamus or ectopic secretion from a neuroendocrine tumour. Chronic excessive GH secretion can lead to a range of effects on soft and bony tissue, most characteristically causing a change in appearance with coarsening of the facial features, broadening of the nose and prominent supra-orbital ridges. There is usually an increase in the soft tissues such as in the hands causing dough-like enlargement and ring and shoe size often increase. Elongation of the jaw leads to dental malocclusion and increased inter-dental spacing. Excessive GH secretion prior to the fusion of bony epiphyses results in gigantism. Accelerated degenerative changes affecting the joints are a common occurrence and major cause of morbidity and although there is an increase in lean body mass and muscle hypertrophy, muscles are weaker (Nagulesparen, Trickey et al. 1976). Carpal tunnel syndrome affects approximately 60% of patients and is believed to be largely due to swelling of the median nerve itself rather than extrinsic compression from increased volume within the carpal tunnel (Jenkins, Sohaib et al. 2000). Hypertrophy of the soft tissues of the upper airway can result in obstructive sleep apnoea. Other common and clinically significant findings include cardiomegaly, hypertension, an increased risk of type 2 diabetes mellitus secondary

to insulin resistance and an increased risk of neoplasia, particularly of the colon. Consequently it is associated with considerable morbidity and mortality; standardised mortality ratios are reported to be 1.3-1.8 with uncontrolled GH excess (Wright, Hill et al. 1970); (Alexander, Appleton et al. 1980); (Bates, Van't Hoff et al. 1993).

Diagnosis of acromegaly

Diagnosis of acromegaly involves the measurement of serum GH, however due to the pulsatile nature of its secretion, a single GH measurement is insufficient. The most commonly used diagnostic test is the oral Glucose tolerance test (OGTT) in which abnormal GH levels fail to suppress following a 75g glucose load. In order to assess the overall GH burden, the mean of a series of 5 samples taken over several hours is calculated (GH day curve or GHDC).

Although a linear correlation exists between log-transformed GH and serum IGF-I, up to one third of patients exhibit discordance between the two measures; a serum IGF-I level alone is therefore insufficient in order to confirm or refute the diagnosis. Assessment of pituitary function and neuro-ophthalmological testing are advisable at diagnosis due to the potential complications of a pituitary mass lesion.

Pituitary MRI is the gold standard radiological investigation to identify the pituitary adenoma; at diagnosis 70% of patients have a macroadenoma measuring more than 10mm and these may invade the cavernous sinus or suprasellar region.

1.3.3 Treatment of Acromegaly

In view of the significant morbidity and mortality of this condition, treatment is needed for all patients. A range of options are available with varying levels of

response and side effects; many patients require more than one modality in order to achieve biochemical cure or control of GH excess.

Pituitary surgery

Trans-sphenoidal surgery is the initial treatment of choice for the majority of patients with cure rates of 70%-90% reported for microadenomas and 30%-50% for macroadenomas. The original trans-sphenoidal operation was performed by Harvey Cushing in 1910 but routine use of this technique only occurred since the mid-1970s when better visualisation techniques became available. In experienced hands the rates of complication are low with mortality reported as less than 0.5%. More recently endoscopic trans-sphenoidal surgery has become a more established technique, associated with improved clearance, less discomfort and fewer complications. The majority of pituitary tumours can be removed via this route, even with extension into the cavernous sinus, thus avoiding the need for the more invasive trans-cranial approaches.

Pituitary Irradiation

Pituitary irradiation is often used as an adjunctive treatment to non-curative pituitary surgery or in the primary treatment of patients unfit for surgery. Traditional external beam pituitary irradiation is focused on the pituitary fossa following accurate dosimetry planning. Most centres use a total dose 4500 cGy in 25 fractions via three fields (one frontal and two temporal). Care must be taken to avoid damage to the optic chiasm with the use of daily fractions of less than 200cGy. Response rates of 50% fall in GH levels in the first two years post irradiation are reported, followed by

a continual exponential decline thereafter (Jenkins, Bates et al. 2006). The majority of patients achieve the target GH levels of less than 2ng/ml over time. 60% of patients are reported to normalise serum IGF-I levels after 10 years; this attenuated response may be due to the chronic low amplitude GH secretion known to generate low-normal IGF-I levels post pituitary irradiation (Toogood, Nass et al. 1997). The main complication of pituitary irradiation is hypopituitarism; at ten years 60% are hypogonadal, 50% ACTH deficient and 40% TSH deficient (Jenkins, Bates et al. 2006).

Stereotactic radiosurgery

Stereotactic radiosurgery using gamma knife radiosurgery or stereotactic multiple arc radiotherapy (SMART) involves the delivery of a single high dose of irradiation, ensuring a rapid reduction in radiation exposure to the surrounding structures. Normalisation of IGF-I is reported to occur in up to 86% of patients at 10 years post GK with discontinuation of GH suppressive medication in 16/40 patients (Landolt, Haller et al. 1998); (Vik-Mo, Oksnes et al. 2007).

Medical treatment for acromegaly

There are three medical options in the treatment of acromegaly; dopamine agonists, somatostatin analogues and the GHR antagonist pegvisomant.

Dopamine agonists

Dopamine agonists (DAs) have been used for the treatment of acromegaly since the mid 1970s (Thorner, Chait et al. 1975). This class of drug causes stimulation of GH

release in normal individuals, but leads to paradoxical suppression of GH hypersecretion in a proportion of patients with acromegaly. Five subtypes of dopamine receptor exist which have specific tissue distribution; D2 receptors are expressed in the anterior and intermediate lobes of the pituitary gland and mediate inhibition of prolactin secretion. The effectiveness of DAs in the control of GH secretion appears to correlate with expression of D2 receptors within the tumour rather than the presence of prolactin (Colao, Ferone et al. 1997). Experience is greatest with bromocriptine; approximately 10% of patients achieve 'safe' GH and normal age-adjusted IGF-I levels using doses substantially higher than those required for the successful treatment of prolactin secreting tumours. Similarly disappointing data exist for pergolide and lysuride, although up to 1/3 of patients achieved biochemical control with the non-ergot derived DA quinagolide at doses 2-4 times those used in the treatment of prolactinomas (Ferrari, Abs et al. 1997). Cabergoline is an ergot derived DA, selective for the D2 receptor with a longer half-life and improved tolerability compared to other DAs such as bromocriptine. In the largest study to date, a normal serum IGF-I was achieved in 39% of patients treated with up to 3.5mg per week (Abs, Verhelst et al. 1998). Prolactin co-secretion and milder disease activity were favourable predictors of a good response; approximately 50% of patients achieved a serum IGF-I within the age-related reference range with starting value <750 ng/ml. My previously published report (Moyes, Metcalfe et al. 2008), detailing the clinical experience in 15 consecutive patients in our centre, demonstrated complete biochemical control (judged by a mean serum GH <5 miu/L and a normal age-adjusted IGF-I level) in 28% of patients using a median weekly dose of cabergoline of 1.75mg (range 0.5-7mg). When assessing serum IGF-I alone, substantial reductions were observed in the majority of patients with 5/15 (33%) achieving normality and a further five patients demonstrating a clinically useful reduction on treatment. Further reports indicate a role for cabergoline as an "add on" for patients partially resistant to somatostatin

analogues (Cozzi, Attanasio et al. 2004); (Selvarajah, Webster et al. 2005); (Gatta, Hau et al. 2005).

Somatostatin analogues

Synthetic somatostatin analogues are have provided an effective and well tolerated medical treatment for a range of neuroendocrine disorders and are particularly helpful in the management of acromegaly. Octreotide can produce suppression of GH for several hours with >90% of patients demonstrating a reduction and 50-60% achieving a GH level of <2ng/ml and normal serum IGF-I after 2 hours. Due to its pharmacokinetics, it requires thrice daily subcutaneous injections. Depot preparations of somatostatin analogues are now in widespread use. These consist of the active drug incorporated with microspheres of biodegradable polylactide and polyglycolide polymers; these allow the slow release of the analogue after intramuscular injection. Two preparations are available octreotide LAR (Sandostatin LAR, Novartis) and lanreotide (Somatuline autogel, Ipsen). Both achieve biochemical control of acromegaly, as evidenced by serum GH <2ngml and normalised IGF-I in approximately 60%-70% of patients (Jenkins, Akker et al. 2000); (Caron, Beckers et al. 2002).

Efficacy of somatostatin analogues is linked to their affinity for somatostatin receptor subtype 2 and 5; these are the receptors through which endogenous suppression of GH occurs and are the predominant receptors found in GH secreting tumours. A significant percentage are resistant to SSA treatment however; this is presumed to be due to reduced receptor subtype 2 or 5 expression in these tumours. In addition to its effects on GH secretion, SSA's have the added benefit of achieving tumour shrinkage in up to 50% of patients; this has led to an increase in its use pre-

operatively to shrink tumour size and potentially improve rates of surgical cure (Carlsen, Lund-Johansen et al. 2008).

Side effects include local inflammation at the site of injection, gastrointestinal symptoms, development of gallstones and although they can improve insulin sensitivity by lowering GH levels, they also exert direct inhibitory effects on pancreatic insulin secretion.

1.3.4 Pegvisomant

Pegvisomant is a genetically engineered analogue of growth hormone (GH), with enhanced affinity for one GHR binding site and reduced affinity to the second GHR binding site, thereby preventing functional dimerization of the GHR (Figure 1.2). Pegylation of this compound, to yield a stable 42-46kDa molecule, results in prolongation of the half-life and reduces immunogenicity (Okada and Kopchick 2001). By blocking effective dimerisation of the GHR, it prevents activation and thereby reduces the production of serum IGF-I. Through this mechanism it has been shown to be extremely effective in the management of acromegaly, with normalisation of serum IGF-I levels reported to occur in 75-97% of patients in a dose dependent manner (Trainer, Drake et al. 2000). It is important to note however that pegvisomant's effect is purely on IGF-I generation due to blockade of the GHR; it has no effect on the tumour itself. Furthermore, there are concerns about the potential for tumour growth due to the lack of negative feedback at the pituitary; two cases have so far been reported. It is difficult however to determine whether this was due to the pegvisomant or whether it is due to the natural history of the tumours themselves; pegvisomant tends to be reserved for patients resistant to other treatment modalities and thus with the potential to be more aggressive.

Increasing clinical experience with pegvisomant has demonstrated a degree of variability in clinical response that is currently unexplained. Doses vary from 10mg to greater than 40mg per day, with marked variation in the IGF-I responses seen. Whilst patient compliance and administration of the subcutaneous injection may influence clinical efficacy, even in the context of standardised clinical trial protocols, variability in response is evident and the underlying reasons are unclear.

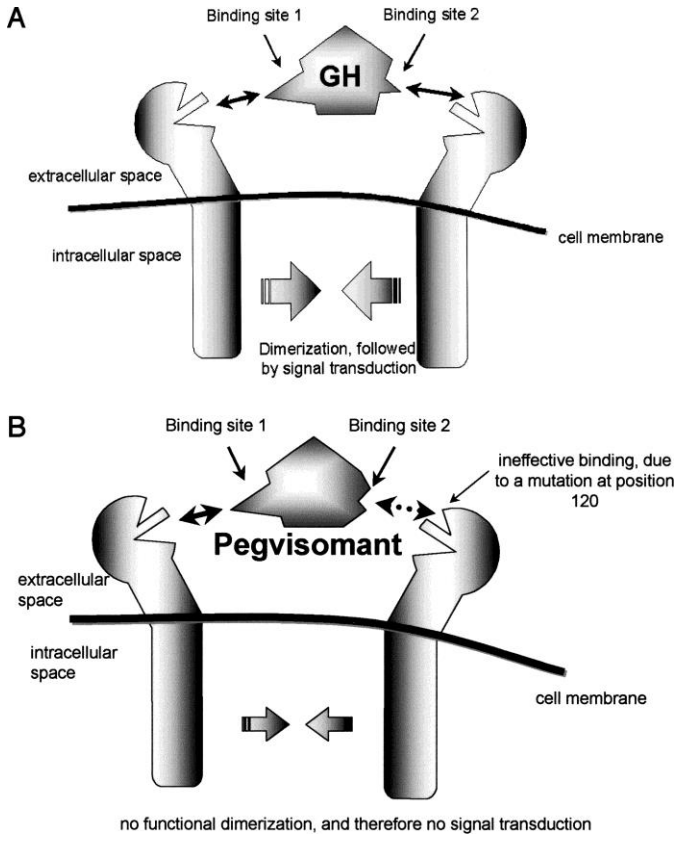
One limitation to the effectiveness of pegvisomant is the lack of accurate targets for the monitoring of treatment. As pegvisomant is structurally similar to GH, standard laboratory assays are unable to distinguish between the two peptides, thereby precluding accurate measurement of GH. Furthermore by blocking activation of the GHR, GH secretion increases with pegvisomant use; serum IGF-I is therefore the only available biochemical marker for GH activity.

For the optimisation of pegvisomant treatment protocols, two questions need to be clarified; what are the underlying reasons for variability in pegvisomant responsiveness and how might we overcome them, Secondly, with pegvisomant use, what should our target serum IGF-I level be to ensure optimise control of GH activity without causing inadvertent GH deficiency.

Figure 1.2 Mechanism of action of Pegvisomant

Taken from Muller et al, Journal of Clinical Endocrinology & Metabolism April 1, 2004 vol. 89 no. 4 1503-1511

This schematic demonstrates the mechanism of action of pegvisomant; A demonstrates the normal binding of GH to the GHR with the following dimerisation and signal transduction. Diagram B represents the binding of Pegvisomant to the GHR, preventing dimerisation and thus signal transduction.



1.3.5 Target for Cure/ Monitoring of Treatment

Several retrospective studies have demonstrated that in acromegaly, lowering of mean serum growth hormone (GH) levels to <5mU/L is associated with restoration of life expectancy to normal (Bates, Evans et al. 1995); (Rajasoorya, Holdaway et al. 1994); (Orme, McNally et al. 1998). Although fewer studies have correlated serum insulin-like growth factor-I (IGF-I) levels with mortality (Swearingen, Barker et al. 1998), measurement of this GH-dependent peptide is increasingly used as the sole marker of disease activity. Consensus guidelines suggest that the goals of treatment of acromegaly should include an epidemiologically 'safe' GH level and a normal age-adjusted serum IGF-I (excluding therapy with pegvisomant, where serum GH cannot meaningfully be measured). A strong linear correlation exists between log-transformed GH and serum IGF-I levels, but significant discordance exists in up to a third of patients; most commonly an elevated age-adjusted IGF-I level in the presence of 'safe' GH values (Freda, Wardlaw et al. 1998)**.** For such patients demonstrating GH/IGF-I 'discordance' clinical decision making is problematic. Evidence exists that higher IGF-I values in the presence of unequivocally 'safe' GH levels are associated with adverse changes in insulin sensitivity (Freda, Wardlaw et al. 1998) but clinical symptoms and signs in this patient group may be absent or subtle and long-term, costly therapy difficult to justify. Understanding the factors that determine this GH/IGF-I discordance could, potentially, facilitate more refined clinical decision making.

1.4 Potential Factors Influencing GH Responsiveness

What factors determine an individual's GH responsiveness in conditions of GH deficiency and excess and also in an individual's response to pegvisomant? A

number of hypotheses exist to explain this variability; one likely factor is variability in the GHR.

1.4.1 Exon 3 deletion of GHR

An obvious candidate for variability in GH responsiveness is the GH receptor (GHR). A genetic polymorphism exists in the GHR resulting in the deletion of exon 3 (d3-GHR) and has been linked to enhanced GH responsiveness in children (Dos Santos C 2004). This is a common polymorphism with an overall prevalence of 25–32% and a homozygosity rate of 9-14% (Pantel, Machinis et al. 2000); (Dos Santos C 2004). It results in the loss of amino acid residues 7-28; the effects of this loss are unknown. Modelling of the residues by crystallography has so far been unsuccessful (Urbanek, Russell et al. 1993); (Sobrier, Duquesnoy et al. 1993) but the peptide is located away from the binding surface of the receptor and does not directly influence the binding of GH to the GHR. It has been speculated that this region may play a role in the conformational changes during activation of the GHR dimer by GH (Dos Santos C 2004).

The d3-GHR polymorphism was first linked to enhanced GH responsiveness in the paediatric population in 2004. GHR signal transduction in transfected cell lines was demonstrated to be 30% higher in d3 homo or heterodimers compared to full length homodimers (Dos Santos C 2004). Furthermore in a cohort of children short for gestational age (SGA) or with idiopathic short stature (ISS) treated with rhGH, 1.7 to 2 times growth acceleration was demonstrated in those possessing one or more d3-GHR allele compared to the full length homozygotes (Dos Santos C 2004). The majority of subsequent studies have been confined to children but data are

conflicting; enhanced linear growth responses to injected GH observed in some, but not all, patients with ISS, SGA, GHD and Turner syndrome (Blum, Machinis et al. 2006); (Binder, Baur et al. 2006); (Carrascosa, Audi et al. 2008); (Jorge, Marchisotti et al. 2006); (Tauber, Ester et al. 2007); (Pilotta, Mella et al. 2006).

Recent studies of adult GHD patients have shown similarly conflicting results. Enhanced IGF-generation was demonstrated after 1 but not 5 years therapy with GH in d3-GHR homo- and heterozygotes, in conjunction with changes in fasting lipid profile (van der Klaauw, van der Straaten et al. 2008). However a more recent study has demonstrated no differences in IGF-I response or reductions in body fat between d3 genotype groups after 1 year of rhGH treatment(Barbosa, Palming et al. 2009) . Data in acromegalic subjects are similarly conflicting; improved efficiency of GH signal transduction into IGF-I production was suggested by one study where lower serum GH levels were detected in the combined d3-GHR group with comparable serum IGF-I levels (Schmid, Krayenbuehl et al. 2007). A subsequent study detected no difference in serum GH or IGF-I between genotype groups at diagnosis, but significantly higher post treatment IGF-I levels were seen in the d3-GHR group compared to fl-GHR, with comparable serum GH levels (Mercado, Gonzalez et al. 2008). More recently, a study of 105 patients with acromegaly demonstrated no difference in serum GH or IGF-I levels between the three genotype groups (fl/fl, fl/d3 and d3/d3) and comparable linear correlation between GH and IGF-I between all 3 groups (Kamenicky, Dos Santos et al. 2009). A summary of the studies of the d3-GHR polymorphism in adult GH and acromegaly populations is shown in table 1.1.

Table 1.1 Summary Table of Previous Studies of the role of d3-GHR in determining an individual's GH responsiveness in Adult Growth Hormone Deficiency

This table summarises previously published studies investigating the role of the d3-GHR polymorphism on GH responsiveness in adult GH deficient patients. Studies varied according to whether they combined the d3-homo and heterozygotes or analysed them separately. Data summarised include the % of each genotype, IGF-I response to GH and any clinical measures used. Clinical results are reported as mean(+/- SD)

Table 1.1

Author/ Year	Cohort	Genotype	ΔIGF-I SDS	rhGH dose (mg/day)	Other clinical measures
Van der Klaauw 2008 (van der Klaauw, van der Straaten et al. 2008)	n= 99 at 1 year n=53 at 5 years	56% fl/fl 38% fl/d3 6% d3/d3	fl/fl vs combined d3 At 1 year 2.1(+/-0.2) vs 3.2(+/-0.3) *p=0.010* At 5 years: 2.6(+/-0.4) vs 2.9(+/-0.4) *p=NS*	1 year: fl/fl:0.4(0.02), d3/d3&fl/d3: 0.4 (0.03) *p=NS* 5 years: fl/fl: 0.5(0.04), d3/d3 &fl/d3: 0.5 (0.05) *p=NS*	**Cholesterol**: 1 yr fl/fl: -0.5(+/-0.1); d3/d3 &fl/d3: -0.08(+/-0.1) *p=0.01* 5 yrs: fl/fl: -0.(+/-0.3); d3/d3 &fl/d3: -0.4(+/-0.2) *p=NS* **WH Ratio** 1 yr fl/fl: -0.004(+/-0.0008) d3/d3 &fl/d3: -0.02(+/-0.009) *p=NS* 5 yrs fl/fl: -0.02(+/- 0.01) d3/d3&fl/d3: -0.03(+/- 0.2) *p=0.03*
Barbosa 2008 (Barbosa, Palming et al. 2008)	n=124 at 1 year	58% fl/fl 42% fl/d3 and d3/d3	fl/fl: M: 3.2(-0.7-7.7) F: 2.1 (0.5-8.8) d3/d3& fl/d3 M: 3.9(0.8-9.1) F: 2.3(-0.6-4.2)	fl/fl M: 0.3 (0.1-0.7) F: 0.5 (0.3-0.9) d3/d3 &fl/d3: M: 0.4(0.3-0.8), F: 0.4 (0.3-0.8)	**ΔBody fat:** fl/fl: M -3.5 (-15 to -10) F: -1/4 (-14 to 3.8) d3/d3&fl/d3 M: -2.4 (-11 to 7.8) F: -3.4 (-7.6 to 8.7)

Table 1.2 Summary of Previous Studies the role of d3-GHR in determining an individual's GH responsiveness in Acromegaly

This table summarises previously published studies investigating the role of the d3-GHR polymorphism on GH responsiveness in adult patients with acromegaly. Studies varied according to whether they combined the d3-homo and heterozygotes or analysed them separately. Data summarised include the % of each genotype, IGF-I response to GH and any clinical measures used. Clinical results are reported as mean(+/- SD)

Table 1.2

Author/ Year	**Cohort**	**Genotype %**	**IGFI**	**GH**
Schmid 2007	n=44	fl/fl 50 d3/d3 &fl/d3 50	fl/fl 670g/l (447-1443) d3 group 840(342-1494) p=0.85	fl/fl 29.7 g/l (3.8-159) d3 group 8.4 (2.6-74) ***p=0.002***
Mercado 2008	n=152	fl/fl 45 fl/d3 32 d3/d3 22	Diagnosis: fl/fl 617ng/ml (SD 151) fl/d3 615 (150) d3/d3 678 (202) *p=0.2* Post Rx fl/fl 367ng/ml (204) fl/d3 497 (212) d3/d3 678 (202) ***p=0.008***	Diagnosis fl/fl 26ng/ml (44.3) fl/d3 24.2 (24.3) d3/d3 41.6 (128) *p=0.1* Post Rx fl/fl 6.8 (24.5) fl/d3 8 (15) d3/d3 6.8 (15.8) *p=0.5*
Bianchi 2009	n= 84	52.4 fl/fl 29.7 fl/d3 17.8 d3/d3	No difference at diagnosis. Post surgery: no difference in IGF-I between genotype groups	No difference at diagnosis Post surgery: fl/d3&d3/d3 significantly lower compared to fl/fl (p=0.22)
			Significantly higher IGF-I GH discordance rates in combined d3 group compared to fl/fl **(p=0.03)**	
Kamenicky 2009	n=105	fl/fl 51 fl/d3 30 d3/d3 19	No difference at diagnosis between fl/fl, fl/d3 and d3/d3	No difference at diagnosis between fl/fl, fl/d3 and d3/d3
			Similar linear correlation between GH and IGF-I between genotype groups (fl/fl: IGFI =377 log10 GH+411, R=0.50, P<0.0001; d3/fl: IGFI=365 log10 GH=391, R=0.53, P<0.001; d3/d3: IGF1=346 log10 GH=448, R=0.46, P<0.05)	

Potential Reasons for Discrepant Results

One possible reason for the discrepancy in findings is variability in the methods used. Carrascosa et al demonstrated that 20% of d3 heterozygotes are misclassified as homozygotes using the standard 3 primer PCR technique (Carrascosa, Esteban et al. 2006); (Carrascosa, Audi et al. 2008) a second 2 primer PCR is required to avoid the preferential production of smaller amplification products. It is also possible that by combining d3 homo and heterozygous groups for analysis, the full effect of the d3-GHR may be wholly or partially concealed.

Potential Therapeutic effect of d3-GHR polymorphism

If the hypothesis that the d3-GHR polymorphism augments GH responsiveness is correct, this may lead to a more personal rhGH dose titration regime with pre-therapeutic genotyping. Possession of the d3-GHR allele/ alleles could potentially require less rhGH to yield the same IGF-I response and require a modified titration regime compared to fl/fl genotypes in GH deficient individuals. Furthermore in the acromegalic population, knowledge of an individual's d3-GHR status could potentially influence how we diagnose and manage such patients. If more IGF-I is generated for the same amount of GH, those possessing the d3-GHR polymorphism could be expected to have more severe IGF-I mediated symptoms of acromegaly compared to the fl/fl population and may require more active treatment in order to lower the IGF-I level.

Other Genetic polymorphisms that may influence GH responsiveness

More recently a further study by Glad et al has investigated the role of six SNPS in the GH receptor and the GH signaling pathway (JAK2, STAT5B, SOCS2 and PIK3CB) in conjunction with the effect of the d3-GHR polymorphism (Glad, Barbosa et al 2013). This demonstrated that after 1 week of GH replacement, fl/fl homozygotes demonstrated a better IGF-I response compared to the combined d3-GHR group (p=0.016). Homozygotes of the minor allele of PIK3CB SNP rs361072 responded better than carriers of the major allele (p=0.025) and both SNPs demonstrated an augmented IGF-I response at 6 months (p=0.041 and p=0.047, respectively) compared to baseline. SNP rs6873545 was associated with an enhanced IGF-I response at 1 year (p=0.041)(Glad, Barbosa et al. 2013).

No other genetic polymorphisms of the GH receptor have been identified which influence GH response, instead focus in the paediatric population has turned to a polymorphism within the IGF-binding protein 3 (IGFBP-3) promoter region (-202 A/C). A study investigating the effect of this polymorphism on circulating IGFBP-3 levels and response to rhGH therapy in pre-pubertal children with GH deficiency, showed that despite similar rhGH doses A allele homozygotes presented higher IGFBP-3 score levels and a higher mean growth velocity in the first year of rhGH treatment compared to A allele heterozygotes and wild type homozygotes (Costalonga, Antonini et al. 2009). No studies of this polymorphism within adult populations have yet been published.

1.4.2 Oestrogen and Pituitary Irradiation

Potential confounders in any analysis of GH responsiveness are the presence of oestrogen and previous pituitary irradiation. Oestrogen is known to attenuate IGF-I production whereas there is evidence from ultrasensitive chemiluminescence assays that patients who have received external pituitary irradiation may generate a low-normal IGF-I level from retained low amplitude GH secretion. Such factors must therefore be accounted for in any statistical analyses.

1.5 Aims of this work

For the two clinical conditions of GH deficiency and acromegaly, there remain a number of unresolved questions; the aim of this thesis is to investigate the following:

1. Is the d3-GHR polymorphism important in determining an individual's GH responsiveness in conditions of GH excess and GH deficiency?
2. Can we tailor the treatment of GH excess and GHD but pre-therapeutically genotyping patients for the d3 polymorphism?
3. For patients treated with Pegvisomant for GH excess, can we optimise the use of serum IGF-I as a marker of disease control?

Chapter 2

Materials and Methods

2.1 Laboratory

2.1.1 DNA Extraction

Genomic DNA was extracted from peripheral blood leucocytes using standard methods (BACC2 DNA extraction kit, GE Healthcare, UK).

- 3mls whole blood mixed with 4x volume reagent A
- Centrifuged at 2500rpm for 5 mins; supernatant discarded.
- Pellet mixed with 2 µl reagent B and vortexed.
- 500 µl sodium perchlorate added and mixed gently
- 2ml chloroform added and mixed gently
- 300 µl resin added and sample centrifuged at 2500rpm for 5 mins
- Supernatant removed and 3x volume cold absolute ethanol added
- DNA strands removed and reconstituted in DNAse free water
- Sample centrifuged for 10 mins at 4000rpm
- Supernatant discarded and pellet washed in 70% ethanol
- Centrifuged for 10 mins at 4000rpm
- Pellet reconstituted in DNAse free water.

2.1.2 Oligonucleotides (Promega)

The following oligonucleotides were used:

Forward: 5’ TGTGCTGGTCTGTTGGTCTG 3’

Reverse full length: 5’ GGATGCTATGTCAGAGTCAG 3’

Reverse d3: 5’ GGTAAGTCACATAGATACTG 3’

How primers were developed

After establishing the gene sequence of interest using internet databases NCBI and ENSEMBL, primer pairs were designed according to the basic principles listed below:

- Primers should ideally be 17-25 bp in length
- Sequences should be non-repetitive and non-palindromic
- G/C content should designed to be 40-60%
- Forward and reverse primers should anneal at approximately the same temperature
- Tm should be between 58 and 68°C and is calculated (approximately) as follows:

- Melting temperature (°C) = (number of C/G bases) x 4 + (number of A/T bases)x 2

- Primers should not form secondary structures

2.1.3 Polymerase Chain Reaction (PCR)

PCR was performed to amplify the region of interest. The basic principle involves denaturing double stranded DNA at high temperatures, ~95°C. This is followed by annealing of sequence-specific oligonucleotide primers typically at temperatures 50-60°C before synthesis of complementary DNA strands from 5' to 3' by a thermostable polymerase. Repeated cycles allow exponential multiplication of a specific portion of DNA.

A PCR reaction mixture, typically in a total volume of 25 µl was as follows:

10x reaction Buffer (Promega, UK)	2.5 µl
10mM dNTPs (Promega, UK)	1 µl
Oligonucleotide (sense) 50 µM	1 µl
Oligonucleotide full length (antisense) 50 µM	0.5 µl
Oligonucleotide d3 (antisense) 50 µM	0.5 µl
Double distilled water	18.8 µl
Taq DNA polymerase (Promega, UK)	0.2 µl
DNA template	1 µl

PCR automated cycling was typically carried out as follows. After the initial denaturation at 95°C for 5 min, followed by 16 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. This was followed by 25 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 1 min, with a final extension step at 72°C for 5 min.

2.1.4 Agarose Gel electrophoresis

All PCR products were run on 1-2% (w/v) agarose gel made in 1X TAE (40 mM Trisacetate, 2 mM disodium ethylenediaminetetraacetate (Na2EDTA), pH 8.3; National Diagnostics, UK), and visualised along side DNA markers (GeneRulerTM

DNA Ladder Mix, 0.5 mg DNA/ml, Fermentas) with ethidium bromide (0.2 µg/ml) staining. Usually 5 µl of each reaction was mixed with loading dye solution (40% w/v sucrose, 0.25%w/v bromophenol blue or Orange G, DEPC water) at a 1:5 ratio prior to loading the wells of a 1% (w/v) agarose gel. Electrophoresis was typically carried out at 120V for 30 min or until clear separation of bands was achieved. Ethidium bromide intercalated into DNA will fluoresce under UV light at 300 nm, allowing the DNA to be visualised. An Uvitec transluminator was used to visualise bands, and capture an image of the resultant gel.

The fl allele is represented by a 521 bp fragment and the d3 allele by a 470 bp fragment.

Repeat PCR

A second PCR reaction was performed on all d3/d3 samples using forward and full length reverse oligonucleotides to confirm homozygosity.

2.2 Biochemical Assays

2.2.1 IGF-I

Between 1997 and 2005 an RIA kit (Diagnostic System laboratories Inc, Webster, TX, USA) with mean intra and inter-assay coefficients of variation (CVs) of 7.2 and 10.4% respectively was used. From 2005- present, serum IGF-I was measured by an automated solid-phase, enzyme-labelled chemiluminescent immunometric assay (Siemens Medical Solutions Diagnostics, Gwynedd, Wales, UK), with intra- and inter-assay CVs of less than 11% and 8% respectively. For each individual patient, baseline, 6 and 12 month IGF-I measurements were performed using the same assay. In order to take into account the change in assay during the time in which these patients were assessed and treated, data in this study have been analysed using standard deviation scores (SDS), calculated as n-mean/SD using age and gender related normative data obtained from our laboratory for each respective assay.

2.2.2 Serum GH

GH was quantitated by an immunoradiometric assay using Immulite 2000 (Siemen's Medical Solutions Diagnostics, Gwynedd, Wales, UK), with inter- and intra-assay CVs of 5%.

2.2.3 Insulin

Immunoradiometric assay using Immulite 2500 (Siemen's Medical Solutions Diagnostics, Gwynedd, Wales, UK) with inter- and intra-assay CVs of 5%.

2.2.4 Glucose

Enzymatic (hexokinase) assay using Roche Modular (Roche Diagnostics GmbH, Mannheim, Germany) with inter- and intra-assay CVs of 2%.

2.2.5 Fibrinogen

Clotting based Claus assay using Sysmex CS2100i (Siemen's Medical Solutions Diagnostics, Gwynedd, Wales, UK) with inter- and intra-assay CVs of 5%.

2.2.6 CRP

Particle-enhanced immunometric assay using Roche Modular (Roche Diagnostics GmbH, Mannheim, Germany) with inter- and intra-assay CVs of 5%.

2.2.7 Lipid Profile

Cholesterol: Enzymatic (cholesterol esterase and cholesterol oxidase) colorimetric assay using Roche Modular (Roche Diagnostics GmbH, Mannheim, Germany) with inter- and intra-assay CVs of 3%.

Triglycerides: Enzymatic (lipoprotein lipase) colourimetric (Trinder endpoint) assay using Roche Modular (Roche Diagnostics GmbH, Mannheim, Germany) with inter- and intra-assay CVs of 3%.

HDL: PEG-modified enzymatic (cholesterol esterase and cholesterol oxidase) colourmetric assay using Roche Modular (Roche Diagnostics GmbH, Mannheim, Germany) with inter- and intra-assay CVs of 2%.

2.2.8 Lipoprotein A

Immunoturbidimetric Assay with intra-assay CV <3% and inter-assay CV <4% (Diazyme, San Diego, USA).

2.3 Clinical Definitions

2.3.1 Severe Growth Hormone Deficiency

The following criteria are used in the diagnosis of severe GHD; all three must be fulfilled to meet the NICE criteria for use of recombinant GH (rhGH).

- Peak growth hormone response of less than 9 mU/litre or 3μg/l on an 'insulin tolerance test' for growth hormone deficiency (or similar validated test such as glucagon stimulation test)
- Demonstrate an impaired quality of life using the validated AGHDA questionnaire, scoring >11/25
- To already be receiving replacement for other deficient hormones if he or she has one or more pituitary hormone deficiencies

2.3.2 Acromegaly

Acromegaly is a condition of uncontrolled growth hormone excess. The gold standard test is the oral glucose tolerance test; confirmation of the diagnosis is made by the failure to suppress serum GH levels to <2mu/l after a 75g glucose load.

2.4 Patient Recruitment

Patients diagnosed and treated for disorders of GH secretion at the department of Endocrinology at St Bartholomew's hospital were invited to participate in these studies; inclusion criteria are listed for each study.

All studies were approved by the local ethics committee and written informed consent was obtained from each patient.

2.4.1 Exclusion Criteria

Patients with identifiable reasons for altered IGF-I production were excluded from analyses. These include the following criteria:

- Renal failure
- Liver failure
- Anorexia
- Opiates
- Levodopa
- DHEA
- Active Cushing's syndrome

2.5 Clinical Measurements

2.5.1 AGHDA

Quality of Life-Assessment of Growth Hormone Deficiency in Adults (QoL-AGHDA) is a validated quality of life measure used to assess the severity of symptoms of severe GHD. These encompass questions regarding symptoms of physical and psychological well being including effects on memory, energy levels and social life. A score of >11/25 is needed to fulfil NICE criteria for severe GHD and for the use of exogenous GH. The AGHDA is reassessed after 9 months rhGH replacement to determine response to treatment; rhGH may be withdrawn unless the AGHDA score has improved by 7 points.

2.5.2 AcroQoL

AcroQoL questionnaire is a disease specific quality of life tool comprising 22 questions on symptoms of physical and psychological well-being in relation to their growth hormone excess.

2.5.3 EuroQoL

EuroQoL is a standardised measure of health status. The visual analogue score comprises a scale of 1-100; the patient marks the level on the scale that corresponds to how well they are feeling.

2.5.4 Waist: Hip Ratio

Waist hip measurements were taken pre-commencement of rhGH and at 6 and 12 month intervals after treatment as a measure of physiological response to rhGH. These measurements were consistently made by the same GH specialist nurse

using the department tape measure, using the narrowest measurement of the waist and the widest hip measurement.

2.5.5 Body fat measurement

Body fat was measured using Dual energy X ray Absorbtiometry (DEXA) and is reported as % body fat.

2.6 Statistics

Expert statistical advice was obtained from Mrs Enid Hennessy, Wolfson Institute, Queen Mary, University of London. All results are reported as mean value ± standard deviation (SD). Statistical significance was accepted at a p value <0.05. All analyses were performed using SPSS (version 11.01; SPSS Inc, Chicago, IL) for Windows XP (Microsoft Corp) and Excel 2007 (Microsoft Corp).

Details regarding the statistical tests used for each study are listed in the relevant chapter.

2.6.1 Standard Deviation Scores (SDS)

Standard deviation scores were calculated to allow direct comparison of IGF-I results obtained from different IGF-I assays. These were calculated as $N - mean / SD$ using age and gender related normative data obtained from our laboratory for each respective assay.

2.6.2 %ULN

Where normative assay data were not available, thereby preventing the calculation of SDS, IGF-I results were reported and analysed as % of the upper limit of the normal age and gender related reference range (%ULN).

Chapter 3

Effect of the d3-GHR polymorphism on rhGH responsiveness in adults with severe growth hormone deficiency

3.1 Introduction

Since the original double-blind placebo-controlled studies of recombinant human growth hormone (rhGH) treatment for adult growth hormone deficiency (GHD)(Jorgensen, Pedersen et al. 1989, Salomon, Cuneo et al. 1989, Binnerts, Swart et al. 1992, Whitehead, Boreham et al. 1992, Bengtsson, Eden et al. 1993), it has become apparent that doses of rhGH need to be individually tailored to patients in order to maximise clinical benefit and minimise the possible risks of prolonged excess GH exposure (Drake, Howell et al. 2001). Most physicians experienced in the management of hypopituitary patients employ a strategy of dose titration, with monitoring of serum insulin-like growth factor-I (IGF-I), a GH dependent peptide, as the main marker of excess dosing. Using such protocols, it is apparent that individual requirements for rhGH vary considerably between patients. For example, in a report of 50 consecutive patients treated with an identical dosing protocol that aims to maintain serum IGF-I between the median and upper end of the age-related reference range, median dose requirements for males and females respectively were 0.8 IU (range 0.4-1.6) and 1.2 IU (0.8-2)Drake, Coyte et al. (1998). Oestrogen is known to attenuate IGF-I production and may partially explain the variability between genders, but this does not explain why dose requirements vary 4- and 2.5-fold in male and female groups respectively (Drake, Coyte et al. 1998).

The factors responsible for determining individual GH responsiveness are currently unknown but an obvious candidate is the GH receptor (GHR). The GHR gene is located on the short arm of chromosome 5 (p13.1-p12) and contains nine coding exons, with exons 3-7 encoding the extracellular ligand binding domain (Leung, Spencer et al. 1987, Godowski, Leung et al. 1989). A genetic polymorphism exists

in the GHR resulting in the deletion of exon 3 and loss of amino acid residues 7-28; the overall prevalence is 25–32% with a homozygosity rate of 9-14% (Pantel, Machinis et al. 2000, Dos Santos, Essioux et al. 2004). The effects of the loss of these amino acids are unknown; modelling of the residues by crystallography has been unsuccessful but it appears not to influence the binding of GH to the GHR in vitro (Sobrier, Duquesnoy et al. 1993, Urbanek, Russell et al. 1993). It has been speculated that this region may play a role in the conformational changes during activation of the GHR dimer by GH (Dos Santos, Essioux et al. 2004).

The presence of one or more d3-GHR alleles has been shown to accelerate linear growth in children short for gestational age (SGA) or with idiopathic short stature (ISS) treated with rhGH (Dos Santos, Essioux et al. 2004), although subsequent data have been conflicting (Blum, Machinis et al. 2006, Jorge, Marchisotti et al. 2006, Carrascosa, Audi et al. 2008, Carrascosa, Audi et al. 2008). In adults with GHD, similarly conflicting results exist. In one study of 99 patients, a greater IGF-I response was demonstrated in subjects carrying at least one d3 allele after 1 but not 5 years of rhGH treatment (van der Klaauw, van der Straaten et al. 2008). However, in a separate, recent report no difference in rhGH responsiveness was demonstrated between d3+ or – genotypes in 124 patients treated for 1 year(Barbosa, Palming et al. 2008). The reasons for these discordant results in adults and children are not clear, although one possibility is that by combining d3 homo and heterozygous groups for analysis the full effect of the d3-GHR may be wholly or partially concealed. The aim of this study was to investigate whether, in the clinical setting, the d3-GHR polymorphism is an important contributor to variable GH responsiveness in adult hypopituitary patients treated with an identical dosing regimen of rhGH. In particular, we were interested to investigate differences in GH responsiveness between d3-GHR homo and heterozygotes as this has not been explored in previous studies.

3.1.1 Aim of this section

- To establish the percentage of patients in our cohort carrying each of the GHR genotypes; fl homozygotes, d3 heterozygotes and d3 homozygotes
- To investigate whether d3- homo or heterozygosity enhances the IGF-I response to rhGH
- To investigate whether rhGH dose requirements decrease due to possession of one or more d3-GHR allele.
- To investigate whether the physiological effects of rhGH vary according to possession of one or more d3-GHR allele.
- To investigate how d3-GHR genotype and potential confounding factors such as pituitary irradiation, influence GH response individually and cumulatively with stepwise regression analyses.

3.2 Study Design

3.2.1 Patient Selection

All patients treated with rhGH for severe GHD within the department of endocrinology at St Bartholomew's Hospital were invited to participate. This study was approved by a local ethics committee (Reference 07/H0701/55) and all patients provided informed, written consent to allow the use of their clinical and biochemical records and all patients provided a 10ml EDTA blood sample to allow GHR genotyping. Details regarding GHR genotyping are given in Methods section 2.1.

194 patients with confirmed severe GHD were recruited. All fulfilled the criteria for the diagnosis of GHD, as per the Growth Hormone Research Society Committee consensus guidelines (Carroll et al, JCEM 1998). The biochemical diagnosis was confirmed by a failure to achieve a peak GH of >9mu/l (3μg/l) during insulin induced hypoglycaemia (nadir glucose ≤2.2mmol/l) or on a glucagon stimulation test.

All had commenced rhGH fulfilling criteria subsequently implemented by NICE (see methods section 2.31) and followed an identical dose titration protocol (Drake et al 1998, J Clin Endocrinol Metab 83, 3913-3919) aiming to achieve and maintain a serum IGF-I between the median and upper end of the age related reference range.

No patients had any identifiable reasons for altered IGF-I production e.g. anorexia nervosa, renal failure, liver failure or concomitant use of opiates, DHEA supplements or levodopa. None had active Cushing's syndrome at commencement of GH; those patients with a diagnosis of Cushing's were 3-22 years post cure/successful control of cortisol excess.

3.2.2 Data Collation

As per the rhGH treatment protocol, after titration of rhGH dose, all patients were routinely assessed clinically and biochemically at 3, 6, 9 and 12 months and annually thereafter. For the purposes of this study, 6 and 12 month clinical data (waist:hip ratio, fasting lipid profile and quality of life AGHDA questionnaire); biochemical response to rhGH treatment (ΔIGF-I SDS) and maintenance dose of rhGH required were all analysed with respect to GHR genotype. To remove the potential confounding effect of rhGH dose, analyses were repeated using the ratio of IGF-I change to rhGH dose (delta IGF-I/ rhGH dose) at 6 and 12 months.

3.2.3 Assays

Please see methods section 2.2

3.2.4 GHR Genotyping

Please see methods section 2.1

3.2.5 Statistical Analyses

Results are reported as mean value ± standard deviation (SD). Statistical significance was accepted at a p value <0.05. One way ANOVA with Fisher LSD post hoc analysis was performed to compare rhGH response at 6 and 12 months and the doses of rhGH required between the 3 genotype groups; fl/fl, fl/d3 and d3/d3. Comparison was also made of the change in biological parameters of GH activity; waist:hip ratio, quality of life (AGHDA) score and fasting lipid profile.

A stepwise multiple regression model was created to assess the individual and cumulative effect of genotype on GH response in conjunction with other potentially contributory factors; GH dose, oestrogen and external pituitary irradiation. To exclude the confounding effect of GH dose, analyses (ANOVA and multiple regression analysis) were performed using the ratio of delta IGF-I/ GH dose at 6 and 12 months. Analysis was performed using SPSS (version 11.01; SPSS Inc, Chicago, IL) for Windows XP (Microsoft Corp).

3.3 Results

3.3.1 Cohort Details

Details regarding the patient cohort and are shown in table 3.1. Out of 194 patients, 81 were male and 113 female, of whom 88 were pre-menopausal or were receiving oestrogen replacement (subcutaneous or oral preparations; numbers were insufficient to allow subgroup analysis). At diagnosis the cohorts were between 8-81 years old. Causes of GHD include pituitary tumours, other intra-cranial tumours such as craniopharyngiomas, pituitary apoplexy, trauma and inflammatory conditions such as lymphocytic hypophysitis. 89% of the cohort had multiple pituitary hormone deficiencies and 11% isolated GHD. 64% had received pituitary irradiation (external beam or gamma knife) as treatment for their underlying condition. The cohort had been treated with rhGH for a mean 91 months, range 12-276 months.

Table 3.1 Clinical features and Demographics of the Cohort studied

This table demonstrates the characteristics of the entire cohort studied, detailing the male to female ratio, including the presence or absence of oestrogen. Further details are given regarding the cause of growth hormone deficiency, the age at diagnosis and whether pituitary irradiation was administered. The confirmation of GHD was made following insulin induced stress test (ITT) and the last row details the duration of rhGH replacement.

Table 3.1

			n= 194	
			N	%
Gender	Male		81	41.8%
	Female	Total	113	58.2%
		-Oestrogen replete	88	45.3%
		-Oestrogen deficient	25	12.8%
Age at Diagnosis		<20	10	5.1%
		21-30	15	7.7%
		31-40	41	21.1%
		41-50	56	28.8%
		51-60	42	21.6%
		>60	30	15.5%
Pituitary Hormone Deficiency	Isolated Growth Hormone Deficiency		20	10.3%
	Multiple Pituitary Hormone Deficiency		174	89.6%
Cause of Growth Hormone Deficiency	Pituitary tumour/ treatment	Total	137	70.6%
		-Non functioning	58	29.8%
		-Prolactinoma	34	17.5%
		-Acromegaly	9	4.6%
		-ACTH secreting	36	18.5%
	Pituitary apoplexy		9	4.5%
	Inflammatory/Infective		9	4.5%
	Trauma		3	1.5%
	Congenital		2	1.0%
	Other CNS tumours		25	12.9%
	Other		8	4.1%
Radiotherapy	Yes	Total	124	63.9%
		External Beam Radiotherapy	122	62.9%
		Gamma Knife Radiosurgery	5	2.6%
	No		69	35.6%
Peak GH on ITT (miu/l)		Mean (SD)	2.36 (2.65)	
Duration of rhGH Treatment		Mean (months)	91	
		Range (months)	12-276	

3.3.2 GHR Genotyping: To determine the percentage of patients in the cohort carrying each of the genotypes; fl/fl, fl/d3 and d3/d3

194 patients were prospectively genotyped to determine the frequency of the d3-GHR polymorphism (see methods section 2.1). DNA was extracted from whole blood and using established oligonucleotides (forward, full length reverse and reverse d3 primers) to identify the region of interest, a PCR reaction was performed and the PCR products identified on agarose gel electrophoresis. The fl allele is represented by a 521 bp fragment and the d3 allele by a 470 bp fragment (figure 3.1a).

A previous study reported an over-estimation of the d3 homozygotes by 20% (Carrascosa, Esteban et al. 2006) due to the misclassification of heterozygotes. This is presumably due to the fact that smaller PCR products are preferentially formed in a competitive reaction. To exclude d3 heterozygosity in d3/d3 homozygotes, the PCR was repeated in all d3/d3 homozygotes using forward and reverse full length oligonucleotides (Figure 3.1b).

The frequencies of the 3 genotypes (fl/fl, fl/d3 and d3/d3) were 52% (n=101), 38.7% (n=75) and 9.3% (n=18) respectively and were in Hardy-Weinberg equilibrium. These frequencies are in accordance with previously published data. Repeat PCR of d3/d3 patients using forward and full length reverse primers resulted in reclassification of 2/20 (9%) patients as heterozygotes. Proportions of male and female subjects were comparable between genotype groups.

The cohort was divided into 3 genotype groups for further analyses; full length homozygotes fl/fl, d3 heterozygotes fl/d3 and d3 homozygotes d3/d3 (Figure 3.1).

Figure 3.1 Amplification products of multiplex PCR

Products are shown on agarose gel stained with ethidium bromide.

The fl allele is represented by a 521 bp fragment and the d3 allele by a 470 bp fragment.

3.1a Lane 1 represents a genomic DNA ladder; lane 2 represents fl/fl homozgote; lane 3 represents d3/d3 homozygote and lane 4 represents fl/d3 heterozygote.

3.1b Result of second PCR to confirm d3-GHR heterozygosity.

This figure shows a comparison of PCR results for the same patient. To avoid the mis-classification of d3 heterozygotes as homozygotes a second PCR reaction was performed using only forward and full length reverse primers.

Lane 1 is a genomic DNA ladder; lane 2 represents the results of a 3 primer PCR demonstrating a single 470bp band (d3/d3); lane 3 represents the same patient's result from a repeat PCR using only forward and full length reverse primers with a 521bp band is visible confirming heterozygosity.

Figure 3.1a

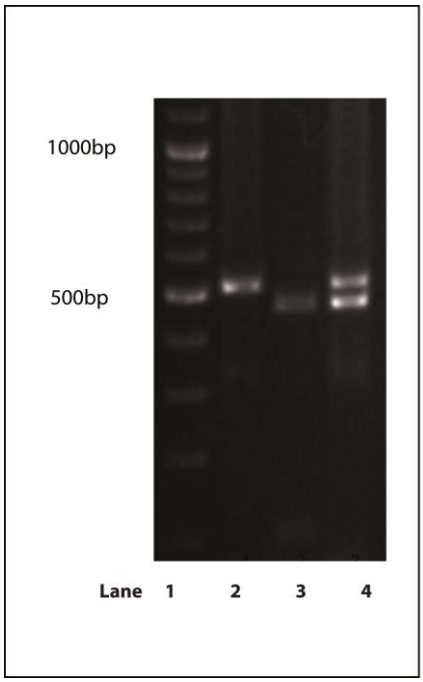
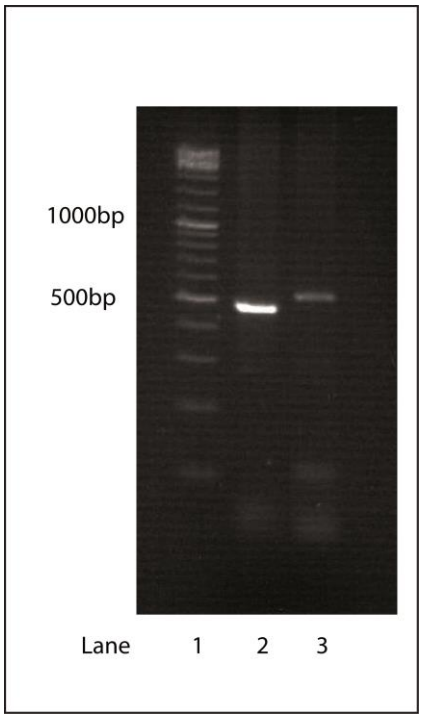


Figure 3.1b



3.3.3 Analyses of IGF-I response in relation to d3-GHR genotype

The working hypothesis for this study was that the presence of one or more d3 allele enhances an individual's response to GH as demonstrated by the amount of IGF-I generated. If correct, one would expect increased IGF-I production for a given level of GH in those patients carrying one or more d3-GHR allele.

3.3.3.1 Baseline Serum IGF-I SDS

To test this hypothesis, a comparison of the baseline IGF-I levels produced by the 3 genotype groups was made using ANOVA with post hoc analyses. This demonstrated that the baseline serum IGF-I SDS results for the 3 genotype groups were comparable; fl/fl mean -1.03 (SD 1.36), fl/d3 -1.14 (SD 1.25) and d3/d3 -1.17 (SD 1.19). No statistically significant difference detected between groups; fl/fl vs fl/d3 p=0.610, fl/fl vs d3/d3 p=0.675 and fl/d3 vs d3/d3 p=0.910 (Table 3.2).

3.3.3.2 Response to rhGH at 6 months

The effect of GHR genotype on the early phase response to rhGH was measured at 6 months. Analyses of the IGF-I SDS at 6 months revealed no statistically significant difference between the three genotype groups (Table 3.2). Analyses of the change in IGF-I (ΔIGF-I) between baseline and 6 months rhGH replacement also demonstrated no statistically significant difference between genotype groups (table 3.2, figure 3.2).

The change in IGF-I per unit rhGH dose was also analysed (ΔIGF-I/rhGH dose) and a comparison of the three genotype groups made; this confirmed a lack of any statistically significant difference in ΔIGF-I/rhGH dose between genotypes with one or more d3 allele and full length homozygotes (Table 3.2, figure 3.3)

Table 3.2 Comparison of the three GHR genotypes for GH responsiveness after 6 months rhGH replacement

This table demonstrates the numbers of patients and the male to female distribution within each genotype cohort; full length homozygote fl/fl, d3 heterozygote fl/d3 and d3 homozygote d3/d3. The left half of the table details the results for each genotype group for IGF-I SDS at baseline and after 6 months rhGH replacement, reporting the mean and (standard deviation) for each cohort. GH responsiveness is reported as ΔIGF-I and ΔIGF-I per rhGH dose (ΔIGF-I/rhGH dose) achieved after 6 months rhGH replacement.

The right hand side of the table shows the results for the comparison between the genotype groups for the above measures using ANOVA with Fisher LSD post hoc analyses. Statistical significance is taken at $p \leq 0.05$. Significant results are highlighted in **bold**.

Abbreviations: fl/fl: full length homozygotes; fl/d3: d3 heterozygotes; d3/d3: d3 homozygotes; rhGH: recombinant growth hormone

Table 3.2

		fl/fl n=101	fl/d3 n=75	d3/d3 n=18		ANOVA p values		
Male		43	32	6		fl/fl vs fl/d3	fl/fl vs d3/d3	fl/d3 vs d3/d3
Female		58	43	12				
IGF-I SDS	Baseline	-1.03 (1.36)	-1.14 (1.25)	-1.17 (1.19)		*0.610*	*0.675*	*0.910*
	6 months	0.82 (1.68)	0.78 (1.60)	0.52 (1.55)		*0.883*	*0.478*	*0.545*
∆IGF-I at 6 months		1.85 (1.88)	1.91 (1.49)	1.69 (1.63)		*0.805*	*0.721*	*0.623*
∆IGF-I/rhGH dose at 6 months		4.98 (11.1)	5.89 (6.89)	5.20 (5.03)		*0.784*	*0.786*	*0.671*

Figure 3.2 Box Plots to compare the three genotype groups for ΔIGF-I between baseline and 6 months rhGH replacement

This box plot represents the results for ΔIGF-I achieved after 6 months rhGH replacement for the three genotype groups; fl/fl, fl/d3 and d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the "whiskers". Occasional outliers are shown as triangles.

Comparison of the three genotype groups were performed using ANOVA with Fisher LSD post hoc analyses and the results are shown above the box plots; NS denotes no statistically significant difference was found.

Figure 3.2

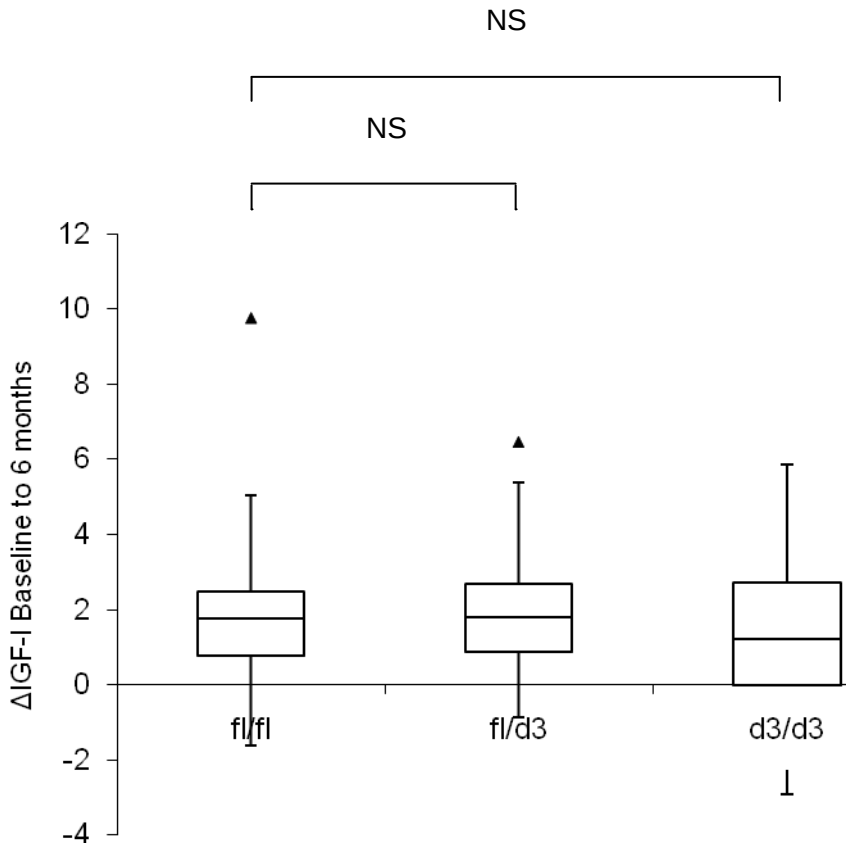
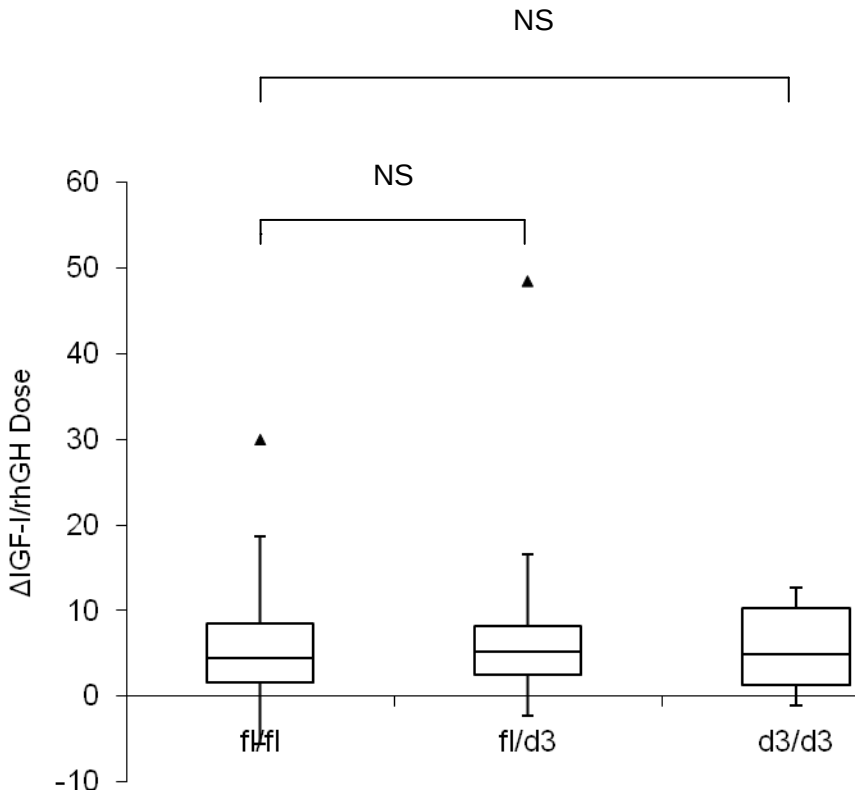


Figure 3.3 Box Plots to compare the three genotype groups for ΔIGF-I/rhGH dose achieved between baseline and 6 months

This box plot demonstrates the change in IGF-I per unit rhGH dose (ΔIGF-I/rhGH dose) at six months for the three genotypes; fl/fl, fl/d3 and d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers” and occasional outliers are shown as triangles.

A comparison between the full length homozygous group (fl/fl) and the d3 homo and heterozygotes (d3/d3 and fl/d3) for ΔIGF-I/rhGH dose was performed using ANOVA with post hoc analyses. No statistically significant difference was detected in these comparisons; NS denotes non-significance.

Figure 3.3



3.3.4 Response to rhGH at 12 months

The longer term response to rhGH was measured at 12 months and results compared between the three genotype groups. The working hypothesis for this section was that the genotype groups possessing one or more d3 allele would confer a greater IGF-I response to rhGH.

A significantly greater IGF-I SDS level at 12 months was achieved in the d3 homozygous group compared to the full length homozygous group using ANOVA with post hoc analyses; (Table 3.3). There was no detectable difference however between the full length homozygous group and the d3 heterozygous group. Furthermore no statistically significant difference was found comparing the d3 homo and heterozygous groups (p=0.066) (Table 3.3).

Similarly, there was a statistically significant difference in ΔIGF-I achieved after 12months rhGH in the d3 homozygous group compared to the full length homozygotes (table 3.3, figure 3.4). No difference was detected between the d3 heterozygotes and full length homozygotes, or between the two d3 possessing groups.

In accordance with the results for ΔIGF-I results achieved after 12 months, a significant difference in ΔIGF-I/rhGH dose was detected in the d3 homozygous group (p=0.004) compared to the full length homozygous group. However a significant difference was also detected in the comparison of the d3 homo and heterozygous groups (p=0.007). There was no detectable difference in ΔIGF-I response comparing the full length homozygotes to the d3 heterozygotes (p=0.882) (Table 3.3, figure 3.5).

Table 3.3 GH Responsiveness after12 months rhGH Replacement

This table demonstrates the numbers of patients and the male to female distribution within each genotype cohort; full length homozygote fl/fl, d3 heterozygote fl/d3 and d3 homozygote d3/d3.

The left half of the table details the results for each genotype group for IGF-I SDS at baseline and after 12 months rhGH replacement, reporting the mean and (standard deviation) for each cohort. GH responsiveness is reported as ΔIGF-I and ΔIGF-I per rhGH dose (ΔIGF-I/rhGH dose) achieved after 12 months rhGH replacement.

The right hand side of the table shows the results for the comparison between the genotype groups for the above measures using ANOVA with Fisher LSD post hoc analyses. Statistical significance is taken at $p \leq 0.05$. Significant results are highlighted in **bold**.

Abbreviations: fl/fl: full length homozygotes; fl/d3: d3 heterozygotes; d3/d3: d3 homozygotes; rhGH: recombinant growth hormone

Table 3.3

		fl/fl n=101	fl/d3 n=75	d3/d3 n=18		ANOVA p values		
Male		43	32	6		fl/fl vs fl/d3	fl/fl vs d3/d3	fl/d3 vs d3/d3
Female		58	43	12				
IGF-I SDS	Baseline	-1.03 (1.36)	-1.14 (1.25)	-1.17 (1.19)		*0.610*	*0.675*	*0.910*
	12 months	0.96 (1.49)	1.01 (1.61)	1.76 (1.52)		*0.843*	***0.046***	*0.066*
ΔIGF-I at 12 months		1.99 (1.49)	2.15 (1.74)	2.93 (1.29)		*0.556*	***0.028***	*0.070*
ΔIGF-I/rhGH dose at 12 months		5.80 (5.58)	5.93 (5.65)	10.2 (8.68)		*0.882*	***0.004***	***0.007***

Figure 3.4 Box Plots to compare the ΔIGF-I achieved between baseline and 12 months rhGH for the three genotype groups

This box plot demonstrates the change in IGF-I achieved after 12 months rhGH replacement for the three genotype groups; fl/lf, fl/d3 and d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers”. Occasional outliers are shown as triangles. Comparison of these results was performed using ANOVA with post hoc analyses.

A significant difference was detected comparing the d3/d3 to the fl/fl group. No significant difference was detected for the d3 heterozygous group compared to the full length homozygous group. No difference was detected between the d3 homo and heterozygous groups.

*denotes statistical significance ($p < 0.05$); NS denotes no statistical significance.

Figure 3.4

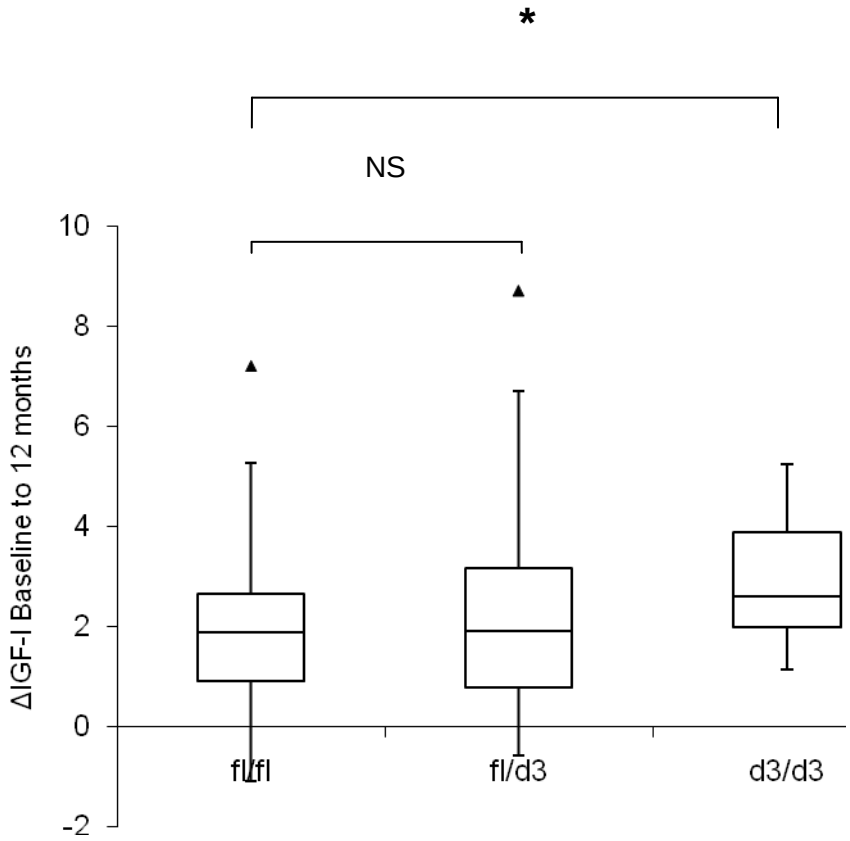
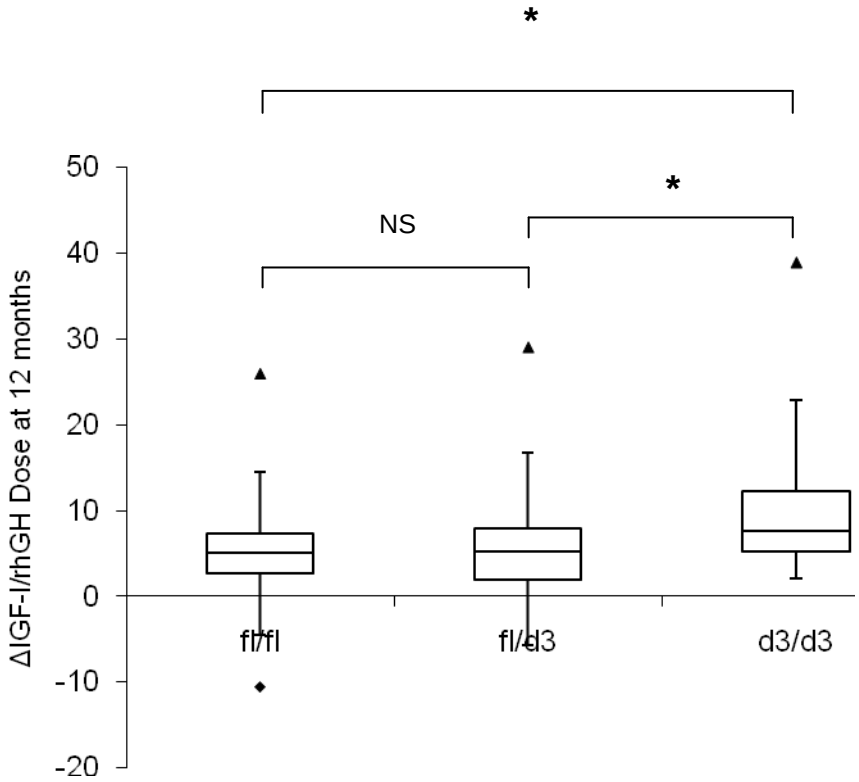


Figure 3.5 Box Plots to compare the three genotype groups for ΔIGF-I/rhGH dose achieved between baseline and 12 months.

This box plot demonstrates the change in IGF-I per unit rhGH dose (ΔIGF-I/rhGH dose) at twelve months for the three genotypes; fl/fl, fl/d3 and d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the "whiskers" and occasional outliers are shown as triangles and diamonds. The results of the ANOVA analyses comparing the fl/fl group with the d3 homo and heterozygous groups are shown; * denotes a statistically significant difference detected between the d3/d3 and fl/fl group and also between the d3/d3 and fl/d3 group. NS denotes no statistical significance detected between the fl/fl and fl/d3 groups.

Figure 3.5


*
*
NS
50
40
30
20
10
0
-10
-20
ΔIGF-I/rhGH Dose at 12 months
fl/fl
fl/d3
d3/d3

3.3.5 Comparison of rhGH Dose Requirements between GHR genotype groups

The working hypothesis for this section is that subjects possessing one or more d3 alleles require a lower dose of rhGH to achieve and maintain a serum IGF-I level within the target range. Analyses were performed of the rhGH doses used at 6 and 12 months for the three genotype groups.

The rhGH doses required to maintain a serum IGF-I between the median and upper limit of the normal reference range at 6 months were comparable between genotype groups; mean 0.37mg, 0.38mg and 0.37mg for fl/fl, fl/d3 and d3/d3 respectively (table 3.4). ANOVA with post hoc analyses confirmed that there was no statistically significant difference between the groups; p=0.802 comparing fl/fl to fl/d3, 0.953 comparing fl/fl to d3/d3 and 0.903 comparing fl/d3 to d3/d3 (table 3.4, figure 3.6).

Furthermore, the doses of rhGH were comparable between genotype groups at 12 months; mean 0.41mg for fl/fl, 0.41mg for fl/d3 and 0.43mg for d3/d3. No statistically significant difference was detected between genotype groups (table 3.4, figure 3.7).

Table 3.4 Comparison of rhGH Dose Requirements between GHR genotype groups

This table demonstrates the mean, (standard deviation) and range of rhGH doses used by each genotype at 6 and 12 months. The right half of the table shows the results of the ANOVA analyses comparing the three genotypes. Statistical significance is taken as p≤0.05.

		fl/fl n=101	fl/d3 n=75	d3/d3 n=18		ANOVA p values		
Gender	Male	43	32	6		fl/fl vs fl/d3	fl/fl vs d3/d3	fl/d3 vs d3/d3
	Female	58	43	12				
rhGH Dose (mg)	6 months	0.37 (0.17)	0.38 (0.16)	0.37 (0.13)		*0.802*	*0.953*	*0.933*
	Range	0.1-1.0	0.1-0.9	0.1-0.7				
	12 months	0.41 (0.22)	0.41 (0.23)	0.43 (0.33)		*0.906*	*0.701*	*0.658*
	Range	0.1-1.2	0.1-1.3	0.1-1.5				

Figure 3.6 Box Plots to compare the three genotype groups rhGH dose at 6 months

This box plot demonstrates the rhGH doses required by the three genotype groups (fl/fl, fl/d3 and d3/d3) at 6 months. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers” and occasional outliers are shown as triangles.

The rhGH doses required by d3 homo and heterozygotes were compared to the full length homozygous rhGH dose requirements using ANOVA with Fisher LSD post hoc analyses. No statistically significant difference was detected in these comparisons; NS denotes non-significance.

Figure 3.6

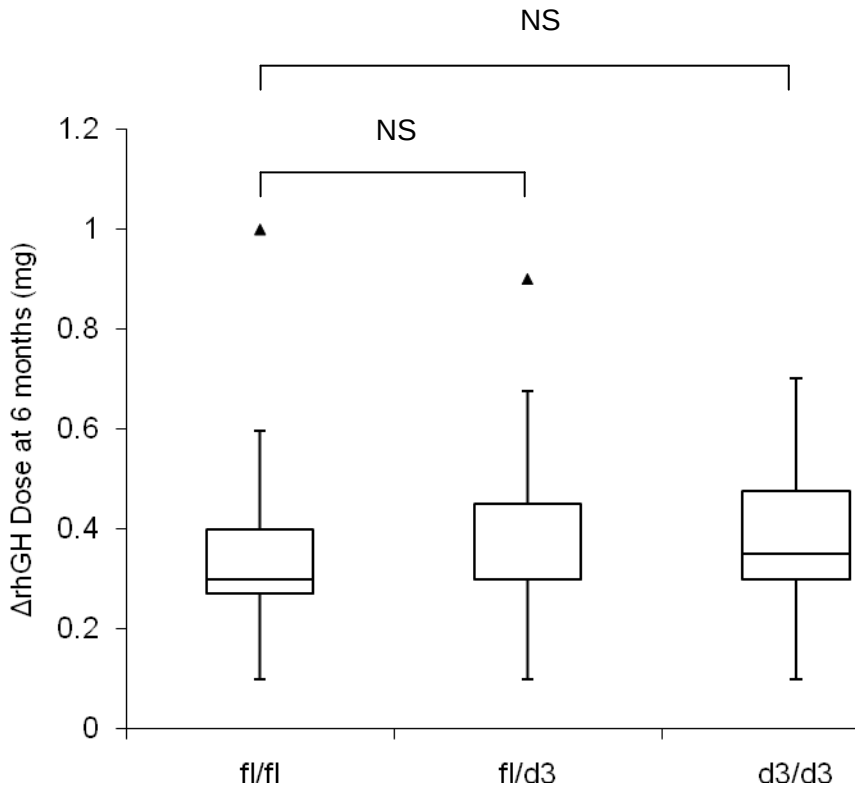
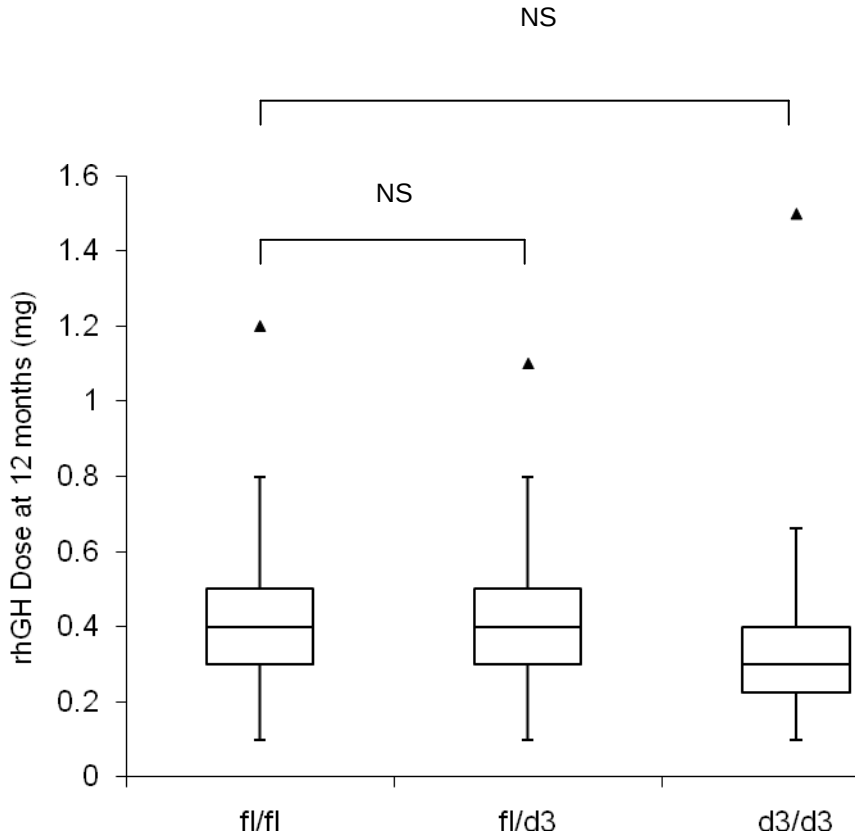


Figure 3.7 Box Plot to compare the rhGH dose requirements for the three genotype groups at 12 months

This box plot demonstrates the rhGH results at 12 months for the three genotype groups; fl/fl, fl/d3 and d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median (the median in this case is at the same level as the lower end of the range) with the range demonstrated by the "whiskers" and occasional outliers are shown as triangles. The results of ANOVA analyses are shown above the diagram; comparison between the fl/fl group and the d3 homo and heterozygotes yielded non-significant results.

NS denotes no statistical significance.

Figure 3.7


NS
NS
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
rhGH Dose at 12 months (mg)
fl/fl
fl/d3
d3/d3

3.3.6 The effect of d3-GHR genotype on clinical markers of rhGH responsiveness

By analogy with the measurement of linear growth in children, certain clinical markers of GH activity are used in the investigation of GH responsiveness in adults. These include quality of life scores, waist: hip ratio and fasting lipid profile.

The hypothesis of this part of the study is that possession of one or more d3 allele conferred an augmented GH response, as demonstrated by the clinical measurements.

3.3.6.1 QoL-AGHDA

For the purposes of this study, AGHDA results were collated after 12 months rhGH treatment and the change in AGHDA (ΔAGHDA) between baseline and 12 months was calculated. The hypothesis for this section is that d3 homo or heterozygosity enhances the clinical response to rhGH replacement, thereby resulting in a greater change in AGHDA score.

The mean ΔAGHDA was 10.3 (SD 6.66) for the fl/fl group, 9.35 (SD 6.00) for fl/d3 and 11.3 (SD 5.75) for the d3/d3 group. Comparison between the three genotype groups for ΔAGHDA using ANOVA yielded no significant difference; p=0.533 for fl/fl vs fl/d3, p=0.472 for fl/fl vs d3/d3 and p=0.296 for fl/d3 vs d3/d3 (Table 3.5, Figure 3.8).

3.3.6.2 Waist: Hip ratio

The hypothesis for this section is that possessing one or more d3 allele enhances the physical response to GH, as demonstrated by an augmented change in waist: hip ratio measurements. For the purposes of this study, the difference in waist: hip

ratio taken at baseline and after 12 months rhGH replacement was calculated. The results were as follows: fl/fl mean (SD) 0.02 (0.16), fl/d3 0.57 (0.16) and d3/d3 0.03 (0.08).

Using ANOVA with post hoc analyses, no statistically significant difference was detected between genotype groups for Δwaist:hip ratio; fl/lf vs fl/d3 p= 0.116, fl/fl vs d3/d3 p= 0.749 and fl/d3 vs d3/d3 p=0.547 (Table 3.5, Figure 3.9).

3.3.6.3 Fasting Lipid Profile

Monitoring of fasting lipid profile occurs routinely as part of the rhGH replacement protocol. The change in total cholesterol (ΔChol) and triglyceride levels (∆Trig) that occurred between baseline and 12 months rhGH replacement were calculated for the three genotype groups and compared using ANOVA with post hoc analyses. The hypothesis for this section is that d3 homo or heterozygosity enhances the change in cholesterol level in response to rhGH replacement.

The mean ΔChol was 0.42 (SD 1.26) for fl/fl, 0.605 (SD 1.17) for fl/d3 and 0.64 (SD 1.44) for d3/d3. ANOVA statistical analyses however, did not detect a significant difference between genotype groups; p=0.124 for fl/fl vs fl/d3, p=0.290for fl/fl vs d3/d3, p= 0.901 fl/d3 vs d3/d3 (Table 3.5, Figure 3.10). Similarly, analyses of the ∆triglyceride results, found no statistically significant difference; p=0.993 fl/fl vs fl/d3, p=0.267 for fl/fl vs d3/d3 and p=0.279 for fl/d3 vs d3/d3 (Table 3.5, figure 3.11).

Table 3.5 Results of the Clinical Markers of GH activity

This table demonstrates the response of clinical markers of GH activity resulting from rhGH replacement and measured as part of the rhGH replacement protocol. Results are reported as mean (SD) for each of the threee genotype groups; fl/fl, fl/d3 and d3/d3.

The clinical markers are the change in QoL-AGHDA score (ΔAGHDA), the change in waist: hip ratio (Δwaist:hip) and the change in fasting lipids, in particular total cholesterol and triglycerides (ΔChol and ΔTrig) achieved after 12 months rhGH replacement. The right side of the table details the results of the comparison of the three genotype groups for these clinical markers using ANOVA with post hoc analyses; significance is taken at $p \leq 0.05$.

Abbreviations: fl/fl: full length homozygotes; fl/d3: d3 heterozygotes; d3/d3: d3 homozygotes; rhGH: recombinant growth hormone; AGHDA: QoL-AGHDA questionnaire score out of a maximum of 25; Chol: cholesterol; Trig: triglycerides

Table 3.5

	fl/fl n=101	fl/d3 n=75	d3/d3 n=18		ANOVA *p values*		
Male Gender Female	43 58	32 43	6 12		fl/fl vs fl/d3	fl/fl vs d3/d3	fl/d3 vs d3/d3
∆AGHDA	10.3 (6.65)	9.35 (6.00)	11.3 (5.75)		*0.533*	*0.473*	*0.296*
∆Waist:Hip Ratio	0.02 (0.16)	0.57 (0.16)	0.03 (0.08)		*0.116*	*0.749*	*0.547*
∆Cholesterol mmol/l	0.42 (1.26)	0.605 (1.17)	0.64 (1.44)		*0.124*	*0.290*	*0.901*
∆Triglycerides mmol/l	0.18 (1.13)	0.14 (0.95)	0.38 (0.86)		*0.993*	*0.267*	*0.279*

Figure 3.8 Comparison of the Three Genotype Groups For ΔWaist: Hip Ratio

This box plot demonstrates the difference between the genotype groups in the change in waist hip ratio (ΔWH) achieved after 12 months rhGH replacement.

The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the "whiskers". Occasional outliers are shown as triangles and diamonds.

Using ANOVA with post hoc analyses, no statistical difference was detected between the three genotype groups.

NS denotes no statistical significance.

Figure 3.8

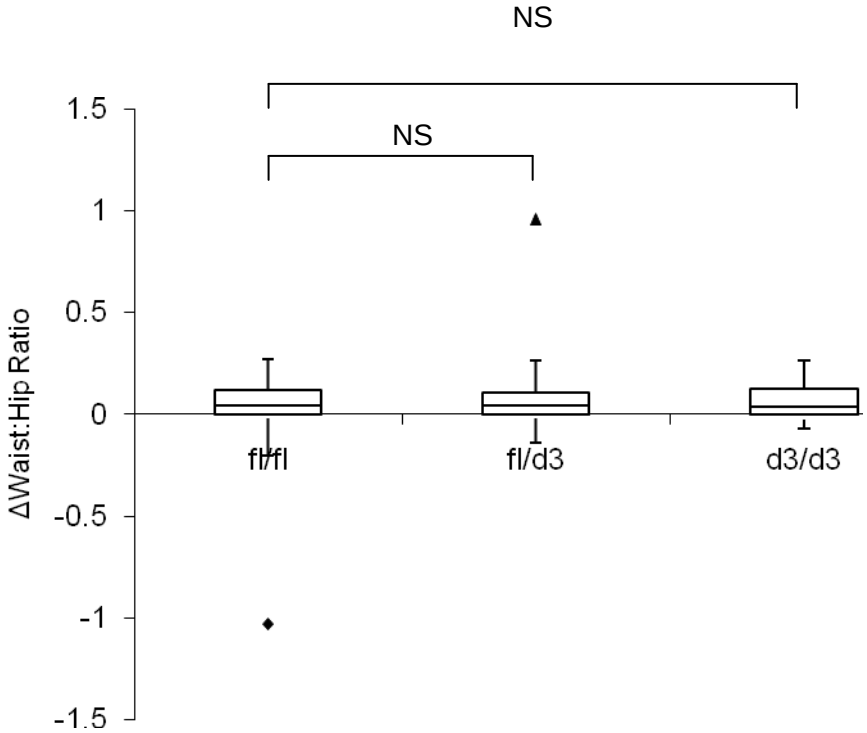


Figure 3.9 Comparison of the Three Genotype Groups For ΔAGHDA Score

AGHDA scores were calculated at baseline and after 12 months rhGH replacement and the change in AGHDA (ΔAGHDA) achieved after 12 months was taken as a measure of GH responsiveness. This box plot demonstrates the ΔAGHDA results for the three genotype groups. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the "whiskers". Occasional outliers are shown as triangles. The results of ANOVA comparison of the three genotypes are shown; NS denotes no statistical significance.

Figure 3.9

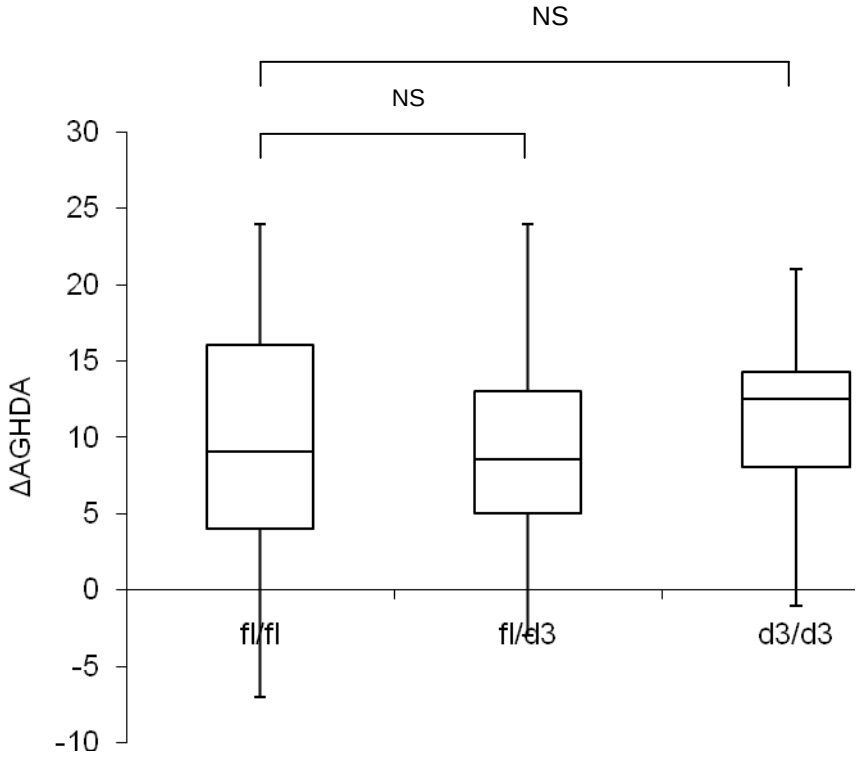


Figure 3.10 Comparison of the Three Genotype Groups for Change in total Cholesterol after 12 months rhGH

This box plot demonstrates the change in total cholesterol (ΔCholesterol) achieved after 12 months rhGH replacement by the three genotype groups; fl/fl, fl/d3 and d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the "whiskers". Occasional outliers are shown as triangles and diamonds. The results of ANOVA with post hoc analyses comparing the fl/fl to the d3 homo and heterozygotes are shown above the diagram; NS denotes no statistical significance.

Figure 3.10

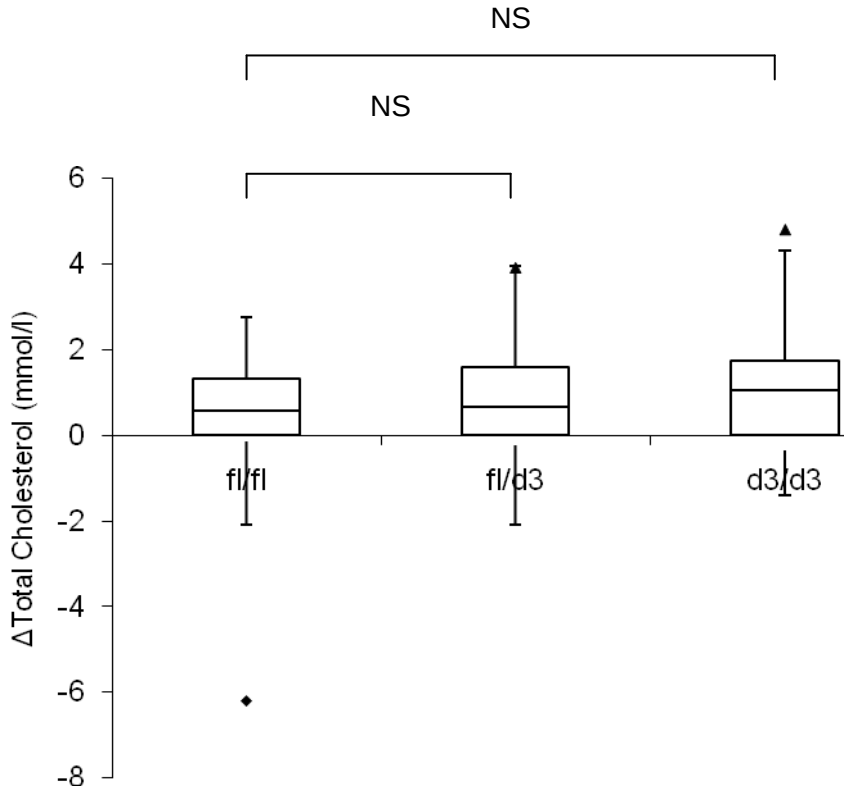
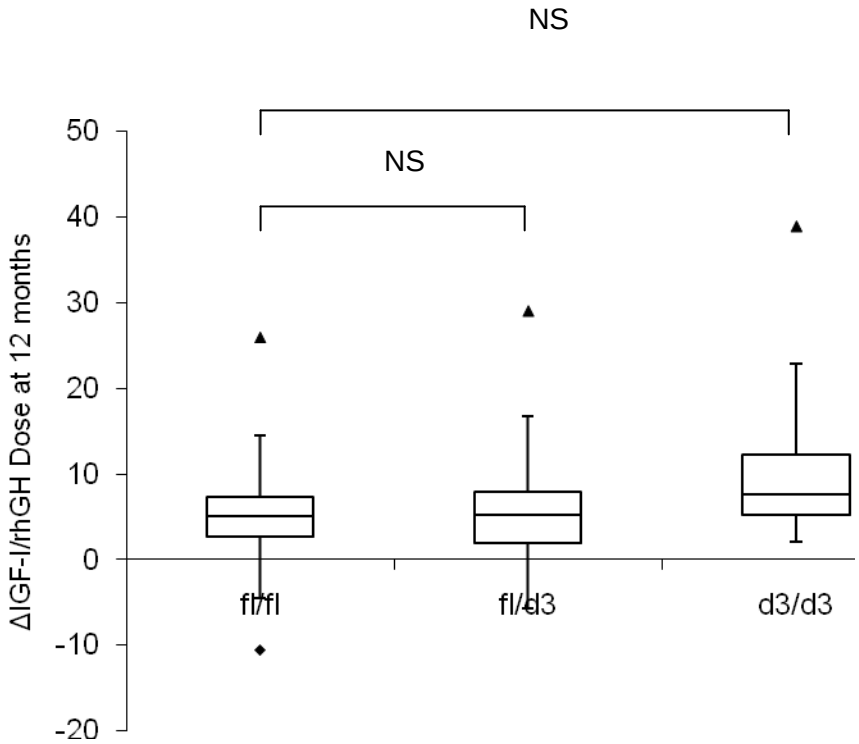


Figure 3.11 Box plot to demonstrate the change in fasting triglyceride level in the three genotype groups.

This box plot demonstrates the change in total triglyceride (Δtriglyceride) achieved after 12 months rhGH replacement by the three genotype groups; fl/fl, fl/d3 and d3/d3.

The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the "whiskers". Occasional outliers are shown as triangles and diamonds. The results of ANOVA with post hoc analyses comparing the fl/fl to the d3 homo and heterozygotes are shown above the diagram; NS denotes no statistical significance.

Figure 3.11


NS
NS
50
40
30
20
10
0
-10
-20
ΔIGF-I/rhGH Dose at 12 months
fl/fl
fl/d3
d3/d3

3.3.7 Multiple Regression Analyses for ΔIGF-I Response

The purpose of this section was to individually and cumulatively investigate the factors affecting GH response as measured by ΔIGF-I response over 6 and 12 months. Using stepwise, multiple regression analyses, known potential confounding variables such as presence of oestrogen and previous pituitary irradiation would be rigorously assessed.

3.3.7.1 ΔIGF-I at 6 months

Neither d3 homo nor d3 heterozygosity conferred an augmented ΔIGF-I response to rhGH between baseline and 6 months when investigated individually or cumulatively; p=0.799 for fl/d3, p=0.755 for d3/d3 on cumulative analyses (table 3.4). There was no significant contribution from rhGH dose (p=0.203) and neither of the potential confounders had a significant effect on ΔIGF-I response at 6 months (p= 0.432 for oestrogen, p=0.231 for pituitary irradiation).

3.3.7.2 ΔIGF-I/rhGH dose at 6 months

To ensure that any potential variations in rhGH dose between genotypes, regression analyses were performed of ΔIGF-I/rhGH dose. Again, these demonstrated no significant relationship of d3 homo or heterozygosity on ΔIGF-I/rhGH dose response between baseline and 6 months (p=0.770 for fl/d3 and p=0.835 for d3/d3). Furthermore, there was no significant effect of either oestrogen or pituitary irradiation; p=0.197 for oestrogen, p=0.144 for pituitary irradiation (Table 3.4).

Table 3.6 Results of Multiple Regression analyses investigating factors influencing GH responsiveness comparing to full length homozygotes (fl/fl) between baseline and 6 months

This table demonstrates the cumulative results of the multiple regression analyses of GH responsiveness as shown by ΔIGF-I and ΔIGF-I per unit dose (ΔIGF-I/rhGH dose). d3-GHR homo and heterozygosity were included in the analyses along with rhGH dose and the potential confounders oestrogen and previous pituitary irradiation.

Statistical significance was taken as p≤0.05.

	Baseline - 6 months			
	ΔIGF-I		ΔIGF-I/Dose	
	β-Coeff	*p value*	β-Coeff	*p value*
fl/d3	0.019	*0.799*	0.022	*0.770*
d3/d3	-0.023	*0.755*	-0.016	*0.835*
Oestrogen (excluding RT)	0.088	*0.432* *(0.473)*	-0.094	*0.197* *(0.136)*
Pituitary Irradiation	0.094	*0.231*	0.107	*0.144*
GH Dose	-0.059	*0.203*	N/A	*N/A*
R Squared	0.020		0.024	

3.3.7.3 Multiple regression results for ΔIGF-I response at 12 months

Regression analyses have shown that d3-GHR homozygosity confers a positive, significant effect of ΔIGF-I response at 12 months; β-coefficient 0.161, p=0.02. d3-GHR heterozygosity however, did not demonstrate any significant effect on ΔIGF-I response; β coefficient 0.053, p=0.477. Further analyses of rhGH dose yielded non-significant results, as did oestrogen and pituitary irradiation (table 3.5).

3.3.7.4 Multiple regression results for ΔIGF-I/rhGH dose response at 12 months

The positive, significant effect of d3 homozygosity was confirmed with analyses of ΔIGF-I per rhGH dose; β coefficient 0.214, p=0.004 (Table 3.5), whereas d3 heterozygosity was again shown to have no significant effect; β coefficient 0.006, p=0.940. Oestrogen appeared to be nearing significance on cumulative analyses with a potential negative effect; β- coefficient -0.122, p=0.089. Further stepwise analyses were performed to determine whether the inclusion or omission of factors such as pituitary irradiation had an effect on the oestrogen analysis result; these confirmed a non-significant result (p=0.072).

Table 3.7 Results of Multiple Regression analyses investigating factors influencing GH responsiveness comparing to full length homozygotes (fl/fl) between baseline and 12 months.

This table demonstrates the cumulative results of the multiple regression analyses of GH responsiveness as shown by ΔIGF-I and ΔIGF-I per unit dose (ΔIGF-I/rhGH dose). d3-GHR homo and heterozygosity were included in the analyses along with rhGH dose and the potential confounders oestrogen and previous pituitary irradiation.

Statistical significance was taken as p≤0.05 and significant results are shown in bold.

	Baseline - 12 months			
	ΔIGF-I		ΔIGF-I/Dose	
	β-Coeff	*p value*	β-Coeff	*p value*
fl/d3	0.053	*0.477*	0.006	*0.940*
d3/d3	0.161	***0.029***	0.214	***0.004***
Oestrogen (excluding RT)	0.06	*0.422* *(0.171)*	-0.122	*0.089* *(0.072)*
Pituitary Irradiation	0.046	*0.528*	0.043	*0.549*
GH Dose	0.142	*0.059*	N/A	*N/A*
R Squared	0.054		0.061	

3.3.8 Results of ΔIGF-I comparing the two IGF-I assays used; pre and post 2005

As two IGF-I assays were used during the course of this study, further analyses were performed of the results taken on each assay in order to investigate whether the change in assay was responsible for the results. Table 3.6 shows the results of the IGF-I results obtained on each assay and for each genotype, and the percentage change in IGF-I was calculated between baseline and 6 months, and baseline and 12 months. Following subdivision of the cohort, numbers were too small for statistical analyses.

3.3.9 Overall Summary of Results

A summary of all of the results for this chapter is given in table 3.7; each of the different measures of GH response are shown and whether d3 homo or heterozygosity significantly influenced GH responsiveness.

Table 3.8 Comparison of the two IGF-I assays used

This table demonstrates the ΔIGF-I response (reported as ng/ml) between baseline and 6 and 12 months respectively, for the two assays used; pre-2005 and post 2005. The left side of the table shows the results for each genotype analysed on each assay demonstrating the ΔIGF-I response between baseline and 6 and 12 months respectively; these are reported as the mean and (SD). The numbers within the subgroups were too small to allow statistical analyses.

Table 3.6

	ΔIGF-I (ng/ml)					
	Assay 1 (pre 2005)			Assay 2 (post 2005)		
	N	0-6 months	0-12 months	N	0-6 months	0-12 months
fl/fl	79	88.8 (71.0)	82.9 (67.5)	22	82.8 (43.3)	89.9 (65.1)
fl/d3	59	106.0 (54.0)	108 (80.0)	16	79.0 (44.1)	93.0 (63.4)
d3/d3	15	85.7 (81.4)	110.3 (69.2)	3	100.0 (30.8)	83.0 (25.9)

Table 3.7 Summary Table of Results for the Effect of d3-GHR on GH responsiveness in GHD cohort

This table summarises all of the results of the statistical analyses performed for each of the measures of GH responsiveness, demonstrating whether there was a detectable difference in the d3-heterozygous and d3-homozygous groups compared to the full length homozygous group.

Table 3.7

Measure	Difference detected d3 Heterozygotes (fl/d3)	Difference detected d3 Homozygotes (d3/d3)
IGF-I SDS Baseline	No	No
IGF-I SDS 6 months	No	No
IGF-I SDS 12 months	No	No
rhGH Dose 6 months	No	No
rhGH Dose 12 months	No	No
ΔIGF-I 0-6 months	No	No
ΔIGF-I 0-12 months	No	Yes
ΔIGF-I/rhGH dose 6 months	No	No
ΔIGF-I/rhGH dose 12 months	No	Yes
ΔAGHDA	No	No
ΔWaist: Hip ratio	No	No
ΔCholesterol	No	No
ΔTriglycerides	No	No

3.4 Discussion

Variability in GH responsiveness is well recognised and has been investigated more extensively in children in whom pre-pubescent linear growth provides an accurate, reliable measure of GH actions. rhGH is frequently used in children with short stature, in particular those short for gestational age (SGA), idiopathic short stature (ISS) and with GH deficiency. The exon 3 deletion of the GHR was originally identified as a potential factor influencing GH responsiveness in the study by Dos Santos et al in 2004 (Dos Santos, Essioux et al. 2004). A 1.7-2 fold growth acceleration was observed in children possessing one or more d3 allele compared to full length homozygotes when treated with rhGH for SGA or ISS. Furthermore, they demonstrated ~30% increased GHR signal transduction in d3 homo or heterodimers compared to full length homodimers (Dos Santos, Essioux et al. 2004). Subsequent studies in paediatric populations have yielded conflicting data and the importance of this polymorphism remains unclear.

GH responsiveness in adult populations is more difficult to establish due to the lack of linear growth as a clinical marker; surrogate markers of GH responsiveness are used instead, such as IGF-I response. Observations from clinical studies and practice suggest there is considerable variability in GH responsiveness in adults; for example rhGH dose requirements vary by 2.5 and 4 fold in male and female populations respectively. If we were able to identify the factors responsible for determining an individual's response to GH, we could tailor rhGH replacement more appropriately and avoid situations of under or over replacement which are associated with morbidity. The purpose of this study was to investigate in detail the role of the exon 3-GHR polymorphism in determining response to rhGH in an adult population with GHD.

Summary of Results

GHR Genotyping

The genotyping of 194 patients with severe GHD from a single centre yielded similar results to those published previously. 52% of the population were full length homozygotes, approximately 38% were d3 heterozygotes and approximately 10% were d3 homozygotes. As previously described, misclassification of d3 heterozygotes as homozygotes was evident in 2 out of 20 subjects; a second PCR was necessary to confirm these results. A 20% misclassification rate was previously demonstrated (Carrascosa, Esteban et al. 2006) and this may be in part responsible for the variability in results published by previous authors.

Analyses of ΔIGF-I response in relation to d3-GHR genotype

There was no detectable difference in ΔIGF-I response achieved by the d3 homo or heterozygotes compared to the full length homozygotes for the early, titration phase of rhGH replacement, as shown by the change over 6 months. Furthermore, correcting for the potential confounding effect of rhGH dose using ΔIGF-I/rhGH dose, also yielded non- significant results. Similarly analyses of the clinical markers of GH activity such as Δwaist hip ratio, also failed to detect a significant difference in response between the d3 possessing genotypes and the full length homozygotes.

These negative findings would indicate that during this early, titration phase of rhGH replacement, there is no difference in GH responsiveness between d3 + or – groups.

Analyses of the results after 12 months rhGH replacement however did reveal a difference in ΔIGF-I response but only in the d3 homozygotes compared to fl/fl; no difference was detected comparing the d3 heterozygotes to fl/fl. Unexpectedly,

analyses of ΔIGF-I per unit rhGH dose demonstrated not only a difference between d3/d3 and fl/fl, but also a significant difference between the d3 homo- and d3 heterozygotes. Many of the previously published studies combined the two groups for analyses, stating that only one d3 allele was sufficient to augment response. These results would suggest otherwise; not only is there are two d3-GHR alleles needed to augment the response, but the behaviour of the d3 homo and heterozygotes are significantly different and should not be combined for analysis.

rhGH Dose

If prior knowledge of an individual's GHR genotype is to be of clinical use, a detectable difference in maintenance rhGH dose requirements would be required; this was not evident on statistical analyses at either 6 or 12 months. The lack of statistical difference in maintenance rhGH dose requirements may be due to the dosing regimen. Patients commenced 0.8IU (more recently 0.3mg) daily, with regular measurement of serum IGF-I and dose adjustments as required, aiming to maintain a value between the median and upper end of the age-adjusted normal range. For the vast majority of male patients, this starting dose resulted in a serum IGF-I within the 'target range', with no requirement for subsequent dose increments. The 'target range' for this dose titration protocol is wide; for example, the normal range for a patient aged 21-30 is 117-358 ng/ml, with a median of 176, thereby giving a 'target range' of 182 ng/ml. Although no increments in dose were needed for the majority of patients above 0.8 or 1.2 IU for males and females respectively, statistically significant differences in IGF-I values at 12 months were noted between genotype groups, but *within* the target range. This would suggest that, although heterogeneity in GH responsiveness between genotype groups is evident at 12

months, this is not of particular clinical relevance and does not explain the marked variation in rhGH doses observed in clinical practice.

Clinical Markers of GH activity

Although a detectable difference in IGF-I response was noted at 12 months, analyses of the clinical markers of GH activity failed to identify a difference in response between the d3+ and d3- groups.

There are a number of potential explanations for this. In the absence of an easily measurable and reproducible measure such as linear growth, the clinical effects of GH in adults are difficult to accurately and measure and are subject to bias.

For example measurement of the waist: hip ratio is dependent on the same person measuring the same sites on two separate occasions with the same tape measure. Similarly the AGHDA questionnaire is a subjective quality of life questionnaire that may be influenced by other non GH related life events.

These results may also be explained by the possibility that the small, albeit detectable, difference in IGF-I generation was insufficient to cause a significant difference in the clinical measures of GH activity; if all patients in the cohort have achieve a target IGF-I of between the median and upper limit of the normal reference range, then variations in serum IGF-I within the reference range may not be associated with observable differences in clinical parameters.

Potential Confounders

Previous studies have identified oestrogen and pituitary irradiation as confounders of any study of the GH/IGF-I axis; oestrogen attenuates the production of IGF-I for a

given level of GH whereas pituitary irradiation in the context of acromegaly is often associated, after many years, with low amplitude, but persistent, GH secretion. Detailed, stepwise regression analyses, however, did not detect a significant effect of either oestrogen or pituitary irradiation on ΔIGF-I at 6 or 12 months. The effect of persistent low grade GH secretion on GH responsiveness is unclear, however it is unlikely that any potential difference between with and without residual GH secretion could be detected in view of the insensitivity of ΔIGF-I and rhGH dose as markers of GH responsiveness.

Subgroup analyses between the groups of women taking different preparations of oestrogen would also be preferable; patches provide a more stable release of oestrogen compared to the peaks and troughs of tablet use. Unfortunately numbers were insufficient to allow subgroup analysis. However, as the overall oestrogen effect was insignificant this issue regarding lack of numbers of a sub-analysis is unlikely to be a major drawback.

In view of the suppressive effect of obesity on IGF-I production, it would also have been preferable to include BMI as a variable in the regression analyses; unfortunately as there was a lack of relevant retrospective data, this was not possible.

Although there may be the possibility of variability in IGF-I response to different preparations of rhGH, we would not anticipate this in view of rhGH being genetically engineered, however this hypothesis was not specifically examined.

In summary, in this study of 194 patients with GHD, which is the largest published to date, the only detectable difference was over 12 months rhGH replacement, and this was only observed in the d3-GHR homozygotes and not d3 heterozygotes. No

differences were found in dose requirements or of the clinical measures of GH activity, thus questioning the clinical relevance of such a finding.

Critique of Work

The strengths of this work lie in the number of subjects included in the analyses which was double that of previously published studies. Robust, methodical, detailed statistical analyses were also performed with input from an expert statistician to ensure the validity of these results. The two d3-GHR groups were analysed separately to ensure that the effect of one or two d3-GHR alleles was fully investigated, and certainly the results of this study would strongly favour the need for this, since a difference in GH response was detected between d3 homo and heterozygotes. Furthermore, following on from the findings of Carracosa et al, repeat PCRs were performed for all labelled d3 homozygotes initially due to the potential for a 20% misclassification rate. The repeat PCR yielded a 10% misclassification rate; considering the potential differences in behaviour of d3 homo and heterozygotes, it raises the question of whether the variability in previously published results are due in part to these misclassifications +/- the combination of the two d3 genotypes for analysis.

One limitation of this work it its retrospective nature; a prospective study would preferable to minimise bias and ensure the validity of the findings however the sheer scale of this study with a cohort of 194 and the methodical way in which identical data sets were collected prospectively during the course of rhGH treatment does help to optimise the validity of the results. Another limitation is the lack of an easily measurable and reproducible physical marker of GH response; paediatric studies benefit from the availability of linear growth to allow robust clinical studies of the

variability in GH responsiveness. All physical measures used as part of the rhGH protocol and for the purposes of this study were subject to bias for example waist:hip ratio will be prone to measurement error even when performed by the same investigator. AGHDA questionnaires are commonly influenced by an individual's personality and judgement of their own symptoms; a more stoic individual tends to score lower marks than a person with depression and yet there is no method for correcting for this when analysing results.

Although changes in fasting lipid profiles can provide a helpful marker of GH activity, a multitude of other influencing factors such as **BMI**, dietary intake and exercise cannot be accounted for and minimised in a retrospective study. The concurrent use of lipid lowering therapy could also influence the reliability of any results of fasting lipid profile both within the cohort but also for each individual's response. Due to the lack of available and accurate data regarding use of lipid lowering therapy over the study period, subgroup analysis was not possible.

It would have been preferable to analyse the effects of varying levels of endogenous and exogenous oestrogen caused by different methods of administration, but the numbers were too small to allow sub-group analyses. One would hypothesise that differing IGF-I responses may occur in those receiving transdermal oestrogen who maintain consistent blood oestradiol levels as opposed to the peaks and troughs experienced with oral oestrogen.

The change in IGF-I assay in 2005 also has the potential to influence results; although all patients used in this study had baseline, 6 month and 12 month IGF-I levels measured on the same assay. The difference between the two IGF-I assays in results and age and gender specified normative reference range precluded direct comparison of results and IGF-I SDS were used for the purposes of this study.

There was also potential for bias from the doses of rhGH used for each individual; to minimise the effect of any potential variability in rhGH prescribing, the change in IGF-I per unit dose was also used for analyses.

Interpretation in the context of published data

Previous studies of the effect of the d3-GHR on GH responsiveness have largely been confined to children. Data have been conflicting, with enhanced linear growth responses to injected GH observed in some, but not all, patients with ISS, SGA, GHD and Turner syndrome (Dos Santos, Essioux et al. 2004, Binder, Baur et al. 2006, Blum, Machinis et al. 2006, Jorge, Marchisotti et al. 2006, Pilotta, Mella et al. 2006, Tauber, Ester et al. 2007). Two recent studies of adult GHD patients have shown similarly conflicting results. Enhanced IGF-I generation was demonstrated after 1 but not 5 years therapy with GH in d3-GHR homo- and heterozygotes, in conjunction with changes in fasting lipid profile (van der Klaauw, van der Straaten et al. 2008) However a more recent study has demonstrated no differences in IGF-I response or reductions in body fat between d3 genotype groups after 1 year of rhGH treatment(Barbosa, Palming et al. 2008) (Barbosa 2008). Conflicting data regarding IGF-I levels in response to a GH stimulus have also been shown in adult acromegalic patients both pre and post-operatively (Schmid, Krayenbuehl et al. 2007) (Mercado, Gonzalez et al. 2008) (Schmid 2007, Mercado 2008).

There are 2 possible reasons for the lack of concordance between studies. First, it is possible that there is variability in the response of d3 homozygotes and heterozygotes; we postulate that this may be due to functional differences in the GHR formed. Assuming equivalent affinity of receptor dimers, only 25% of the

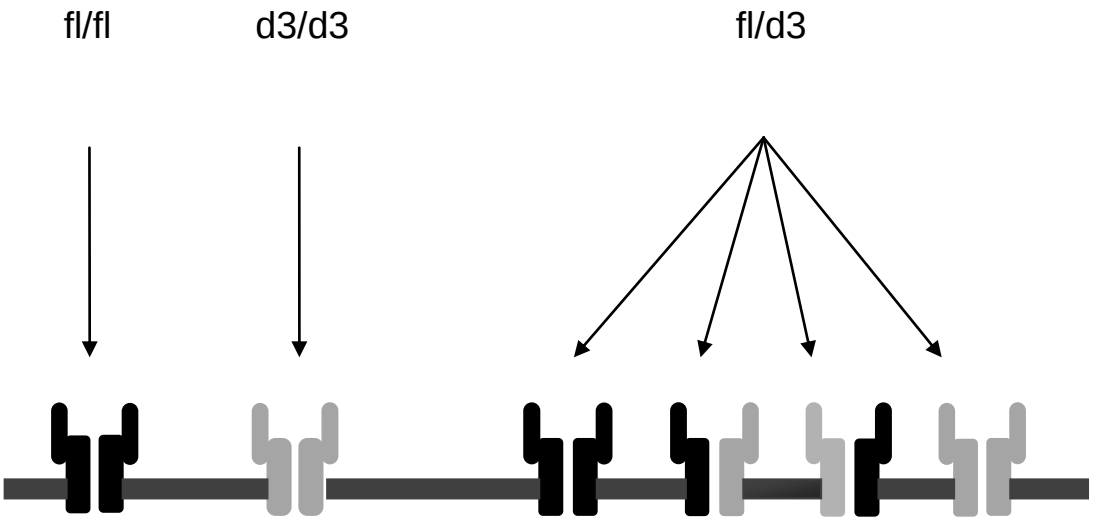
GHRs of heterozygous patients will be d3/d3 homodimers (Figure 3.12). If a d3/d3 homodimer is required for augmented GH responsiveness, then a d3 heterozygote will exhibit only 25% of the 'extra' IGF-I generation capacity: an effect that may not be detected in smaller studies. More detailed studies of the transcriptional activity of hetero versus homodimers are needed to determine whether there is a difference in functional activity and thus whether there is a partial increase in GHR response in d3 heterozygotes in comparison to d3 homozygotes. Second, repetition of the PCR with two primers has ensured that our classification of subjects as d3 homo- or hetero-zygotes is robust; previous studies have shown that 20% of d3 heterozygotes are misclassified as homozygotes without this additional step (Carrascosa, Esteban et al. 2006, Carrascosa, Audi et al. 2008, Carrascosa, Audi et al. 2008). As the majority of previous studies have not performed this second PCR, it is possible that a significant proportion of subjects have been mislabelled. If GH responsiveness varies between d3 homo- and heterozygotes, such genotype misclassifications may have contributed to the inconsistency of published clinical data.

In conclusion this large scale extensive study of the effect of the d3-GHR genotype on rhGH responsiveness in adult hypopituitary patients, has demonstrated an increase in rhGH responsiveness at 12 months. However, given only 10% of the population are homozygous for d3-GHR and the marginal nature of the increased response, this is unlikely to explain the marked variability in rhGH dose requirements in this patient group and further studies are needed before more tailored approaches to rhGH dosing can be developed.

Figure 3.12 d3-GHR Homo- and Heterodimers

Schematic diagram to demonstrate the influence of GHR genotype on the GHR formed. Black represents the full length (fl) and grey represents d3 transmembrane domains; two chains dimerise to form a functional GHR. The type of GHR formed depends on genotype; homozygous individuals for wild-type or d3 alleles form GHR homodimers (fl/fl or d3/d3 respectively). Heterozygous individuals possess a mixture of fl and d3 homo and heterodimers; assuming equal affinity of receptor dimers, only 25% will be d3 homodimers.

Figure 3.12



Chapter 4

Effect of the d3-GHR Polymorphism on rhGH Responsiveness in Adults with Acromegaly

4.1 Introduction

Several retrospective studies have demonstrated that in patients with acromegaly, lowering of mean serum growth hormone (GH) levels to <5mU/L is associated with restoration of life expectancy to normal (Bates 1995, Rajasooriya 1994, Orme 1998)(Rajasoorya, Holdaway et al. 1994, Bates, Evans et al. 1995, Orme, McNally et al. 1998). Although fewer studies have correlated serum insulin-like growth factor-I (IGF-I) levels with mortality (Swearingen 1998), measurement of this GH-dependent peptide is increasingly used by many physicians (particularly in North America) as the sole marker of disease activity. Consensus guidelines suggest that the goals of treatment of acromegaly should include an epidemiologically 'safe' GH level and a normal age-adjusted serum IGF-I (excepting therapy with pegvisomant, where serum GH cannot meaningfully be measured). A strong linear correlation exists between log-transformed GH and serum IGF-I levels, but significant discordance exists in up to a third of patients; most commonly an elevated age-adjusted IGF-I level in the presence of 'safe' GH values(Freda, Wardlaw et al. 1998). For such patients demonstrating GH/IGF-I 'discordance' clinical decision making is problematic. Evidence exists that higher IGF-I values in the presence of unequivocally 'safe' GH levels are associated with adverse changes in insulin sensitivity (Freda 1998) but clinical symptoms and signs in this patient group may be absent or subtle and long-term costly therapy, difficult to justify. Understanding the factors that determine this GH/IGF-I discordance could, potentially, facilitate more refined clinical decision making.

The deletion of exon 3 in the GHR (d3-GHR) has been linked to augmented growth velocity in GH deficient (GHD) children treated with GH. Data in acromegalic subjects are conflicting; this may be in part attributable to variations in methodology such as the grouping together of d3 homo and heterozygotes for analysis. The

purpose of this study was to investigate the effect of the d3-GHR on the IGF-I response to GH in a cohort of patients with acromegaly.

Aim of this section

- To establish the percentage of patients in our cohort carrying each of the GHR genotypes; fl homozygotes, d3 heterozygotes and d3 homozygotes
- To investigate whether d3- homo or heterozygosity influences the IGF-I response to GH

4.2 Study Design

4.2.1 Patient Selection

All patients with acromegaly within the department of endocrinology at St Bartholomew's Hospital (approximately 400 in total) were invited to participate. This study was approved by a local ethics committee (Reference 07/H0701/55) and all patients provided informed, written consent to allow the use of their clinical and biochemical records and all patients provided a 10ml EDTA blood sample to allow GHR genotyping. Details regarding GHR genotyping are given in methods section 2.1.

79 adult patients with acromegaly from a single centre were studied with prospective genotyping of their growth hormone receptor (GHR) and collation of retrospective clinical data. All patients were in routine clinical follow-up and the diagnosis of acromegaly had been confirmed biochemically by a failure to suppress GH levels on a standard oral glucose tolerance test.

No patients had any identifiable reasons for altered IGF-I production e.g. anorexia nervosa, renal failure, liver failure or concomitant use of opiates, DHEA supplements or levodopa.

4.2.2 Data Collation

Data collation was limited by the lack of an available serum IGF-I assay pre-1994; using source data (hospital case notes) diagnostic IGF-I results were only available for 44 of the 79 patients with diagnostic GH results available in 70 out of 79 patients.

Routine assessment of serum IGF-I and the mean of a five point GH “day curve” were performed at three months after the primary form of treatment (surgery, medical therapy, radiotherapy). These results were collated for all 79 patients within the study cohort and used for analysis.

IGF-I levels were converted to % upper limit of the age related normal reference range (IGF-I%ULN) to aid comparison between results. Details regarding the IGF-I and GH assays are given in methods section 2.2.

4.2.3 Statistical Analyses

Expert statistical advice was obtained. Data were assessed for normal distribution; in view of the presence of skewed data, these are reported as median (range) and Kruskall-Wallis non-parametric testing were used for comparison of results.

GH and IGF-I levels were converted to $\log_e$GH and $\log_e$IGF-I respectively to minimise the influence of outliers for the purposes of regression analyses.

Statistical significance was accepted at a p value <0.05. All analyses were performed using SPSS (version 11.01; SPSS Inc, Chicago, IL) for Windows XP (Microsoft Corp) and Excel 2007 (Microsoft Corp).

4.3 Results

4.3.1 Cohort Details

Details regarding the patient cohort are shown in table 4.1. The cohort were aged 18-79 years old at diagnosis and consisted of 41 males and 38 females (Table 4.1). The mean GH level (taken from a 5 point day curve) at diagnosis ranged from 5.1-527.6miu/l, with a mean of 68.3miu/l. Diagnostic IGF-I levels, reported as % upper limit of the normal reference range were 125-700.4% with a mean of 313.5% ULN (Table 4.1).

Pituitary adenomata were evident on diagnostic imaging in 73 patients; 56 (76%) had macroadenomata and cavernous sinus involvement was evident in 11 patients. Four modalities of treatment were used as primary treatment for acromegaly; 49 patients received trans-sphenoidal surgery (TSS), 16 had pituitary irradiation (EBRT), 11 received somatostatin analogues and 3 were treated with dopamine agonists. Using recently defined criteria for cure (mean GH <2miu/l and IGF-I within age-adjusted normal reference range) 20 out of 79 patients achieved remission after primary treatment.

Table 4.1 Clinical features and Demographics of the Cohort

			n= 79	
			N	%
Gender	Male		40	50.6%
	Female	Total	39	49.3%
		-Oestrogen replete	20	25.3%
		-Oestrogen deficient	19	24.0%
Age at Diagnosis		<20	4	5.1%
		21-30	7	8.8%
		31-40	19	24.1%
		41-50	26	32.9%
		51-60	18	22.8%
		>60	5	6.3%
MRI	Macroadenoma		56	70.9%
	Microadenoma		17	21.6%
	Empty Sella		6	7.6%
Surgery			62	78.5%
Radiotherapy	Yes	Total	48	60.8%
		-EBRT	47	59.5%
		-Gamma Knife Radiosurgery	10	12.6%
	No		31	39.2%

4.3.2 GHR Genotyping

79 patients were prospectively genotyped to determine the frequency of the d3-GHR polymorphism (see methods section 2.1). DNA was extracted from whole blood and using established oligonucleotides (forward, full length reverse and reverse d3 primers) to identify the region of interest, a PCR reaction was performed and the PCR products identified on agarose gel electrophoresis. The fl allele is represented by a 521 bp fragment and the d3 allele by a 470 bp fragment (figure 4.1a).

Repeat PCR were performed using only forward and reverse full length oligonucleotides (Figure 4.1b) to avoid the potential misclassification of the d3 heterozygotes as homozygotes, as discussed in section 3.

The frequencies of the 3 genotypes (fl/fl, fl/d3 and d3/d3) were 50.6% (n=40), 36.7% (n=29) and 12.6% (n=10) respectively and were in Hardy-Weinberg equilibrium. These frequencies are in accordance with previously published data. Repeat PCR of d3/d3 patients using forward and full length reverse primers resulted in reclassification of 2/12 (16.7%) patients as heterozygotes. Proportions of male and female subjects were comparable between genotype groups.

The cohort was divided into 3 genotype groups for further analyses; full length homozygotes fl/fl, d3 heterozygotes fl/d3 and d3 homozygotes d3/d3.

Figure 4.1 Amplification products of multiplex PCR

Products are shown on agarose gel stained with ethidium bromide.

The fl allele is represented by a 521 bp fragment and the d3 allele by a 470 bp fragment.

4.1a Lane 1 represents a genomic DNA ladder; lane 2 represents fl/fl homozgote; lane 3 represents d3/d3 homozygote and lane 4 represents fl/d3 heterozygote.

4.1b Result of second PCR to confirm d3-GHR heterozygosity.

This figure shows a comparison of PCR results for the same patient. To avoid the mis-classification of d3 heterozygotes as homozygotes a second PCR reaction was performed using only forward and full length reverse primers.

Lane 1 is a genomic DNA ladder; lane 2 represents the results of a 3 primer PCR demonstrating a single 470bp band (d3/d3); lane 3 represents the same patient's result from a repeat PCR using only forward and full length reverse primers with a 521bp band is visible confirming heterozygosity.

Figure 4.1a

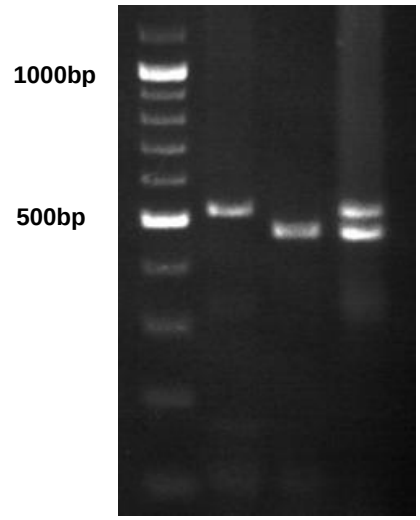
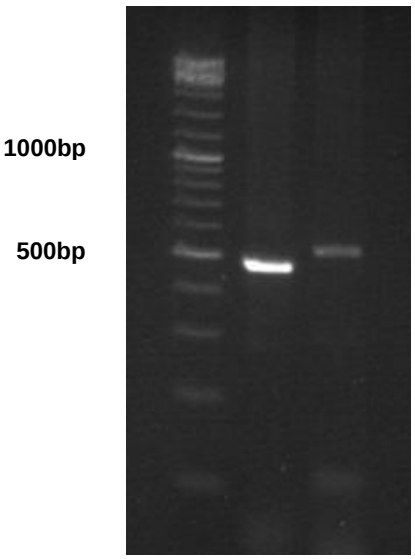


Figure 4.1b



4.3.3 Comparison of IGF-I response in relation to d3-GHR genotype

The working hypothesis for this study was that the presence of one or more d3 allele enhances an individual's response to GH as demonstrated by the amount of IGF-I generated. If correct, one would expect increased IGF-I production for a given level of GH in those patients carrying one or more d3-GHR allele at any given time.

4.3.3.1 Whole Cohort analyses: Post Primary treatment

To test this hypothesis, analyses of IGF-I %ULN and mean GH level (from a 5 point GH day curve) and IGF-I/GH were performed to compare the three genotype groups; fl/fl, fl/d3 and d3/d3. Results for the whole cohort (n=79) were taken from the standard three month post treatment assessment.

As the distribution of GH and consequently IGF-I/GH were skewed, non-parametric tests were performed. IGF-I %ULN levels were 107.9 (19.1-478.5) for fl/fl, 124.5(28.1-423.9) for fl/d3 and 146.8 (48.2-640.5) for d3/d3; no statistically significant difference was detected between genotype groups (p=0.806) (table 4.2a, figure 4.2a). GH levels were median 3.9 (range 0.5-167.4) for fl/fl, 3.16 (0.5-438) for fl/d3 and 8.37 (1.1-110) for d3/d3 (table 4.2a, figure 4.2b); no statistically significant difference was detected between genotype groups (p=0.906). IGF-I/GH were 25.2 (1.6-101.1) for fl/fl, 28.5 (0.44-250.7) for fl/d3 and 23.2 (4.82-58.6) for d3/d3; no statistically significant difference was detected between genotypes (p=0.395) (table 4.2a, figure 4.2c).

Table 4.2 Comparison of the three GHR genotypes for GH and IGF-I levels taken 3 months post primary treatment

This table summarises the GH and IGF-I results for the cohort of 79 patients taken at 3 months after their primary treatment for acromegaly. The left half of the table demonstrates the numbers of patients and the male to female distribution within each genotype cohort; full length homozygote fl/fl, d3 heterozygote fl/d3 and d3 homozygote d3/d3.

The median and (range) for GH, IGF-I and GH/IGF-I are shown for each genotype group. The right hand side of the table shows the results of the statistical analyses comparing the results for the genotype groups; Kruskall Wallis non-parametric analyses were used in view of the skewed data. Statistical significance is taken at $p \leq 0.05$.

Abbreviations: fl/fl: full length homozygotes; fl/d3: d3 heterozygotes; d3/d3: d3 homozygotes; IGF-I %ULN: % upper limit of normal reference range for IGF-I

	fl/fl	fl/d3	d3/d3	*p value*
Number (% Female)	40 (52.5%)	29 (44.8%)	10 (40%)	
IGF-I %ULN	107.9 (19.9-478.5)	124.5 (28.2- 423.9)	146.8 (48.3-640.5)	*0.539*
Mean GH (miu/l)	3.9 (0.5-167.4)	3.16 (0.5-438)	8.4 (1.1-110)	*0.523*
IGF-I/GH	25.2 (1.62-101.1)	28.5 (0.44-250.7)	23.2 (4.82-58.6)	*0.641*

Figure 4.2 Box Plots to compare the three genotype groups for IGF-I post primary treatment

This box plot represents the post treatment results for IGF-I for the three genotype groups; group 1= fl/fl, group 2= fl/d3 and group 3= d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers”. Occasional outliers are shown as circles and asterix.

Comparison of the three genotype groups was performed using non-parametric testing; no statistically significant difference was detected between the genotype groups.

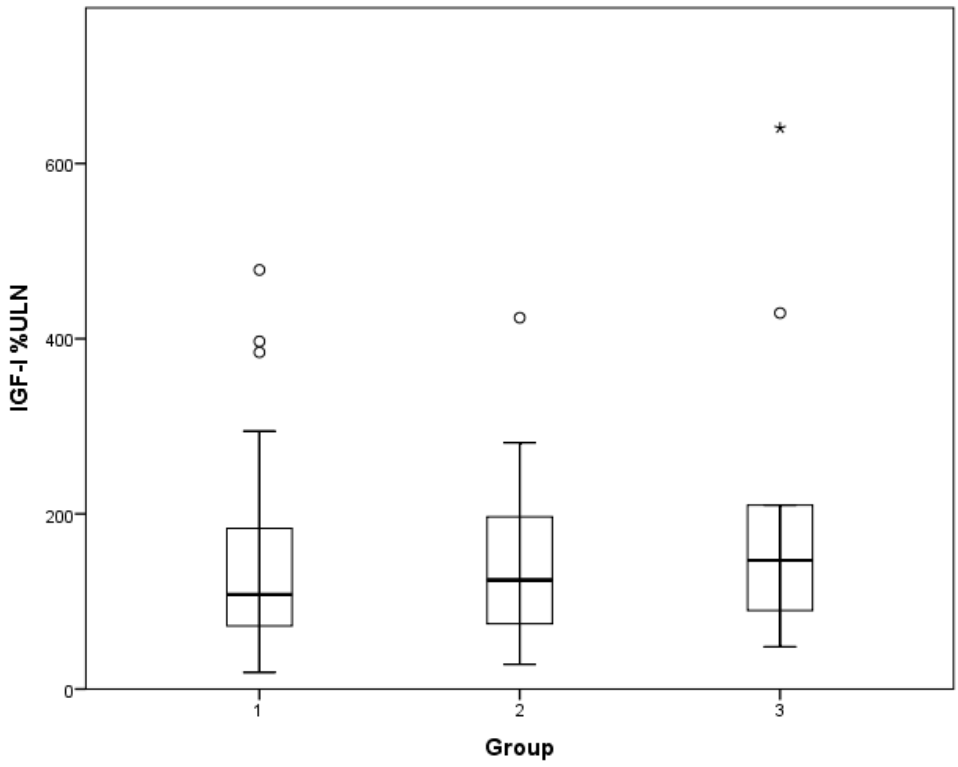


Figure 4.3 Box Plots to compare the three genotype groups for GH post primary treatment

This box plot represents the post treatment results for GH for the three genotype groups; group 1= fl/fl, group 2= fl/d3 and group 3= d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers”. Occasional outliers are shown as circles and asterix.

Comparison of the three genotype groups was performed using non-parametric testing; no statistically significant difference was detected between the genotype groups.

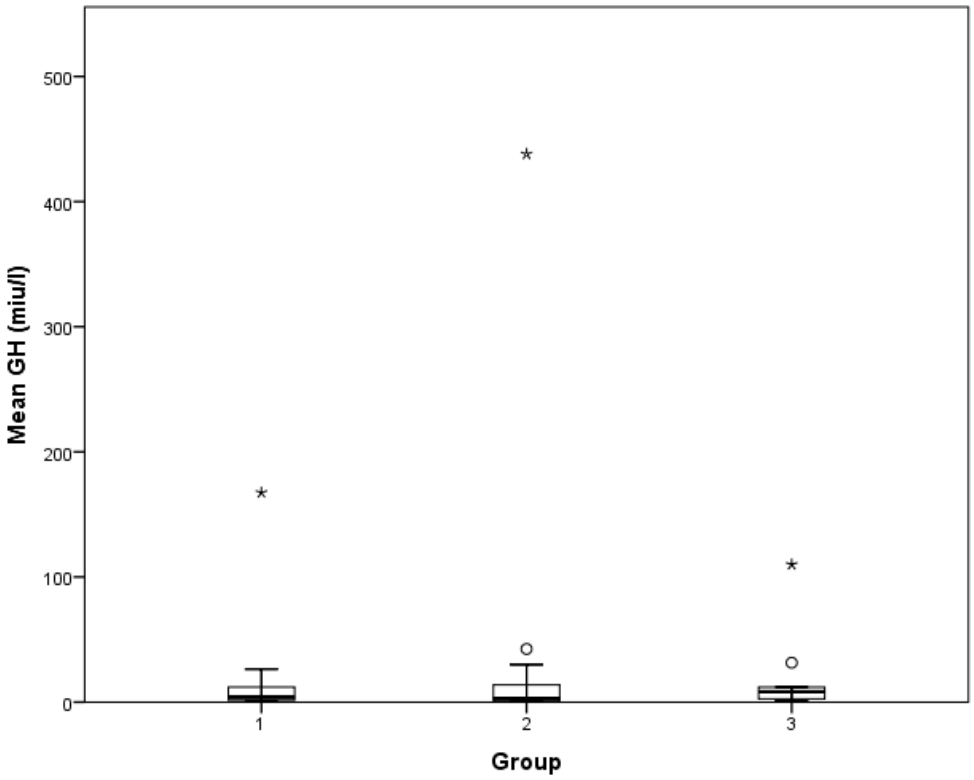
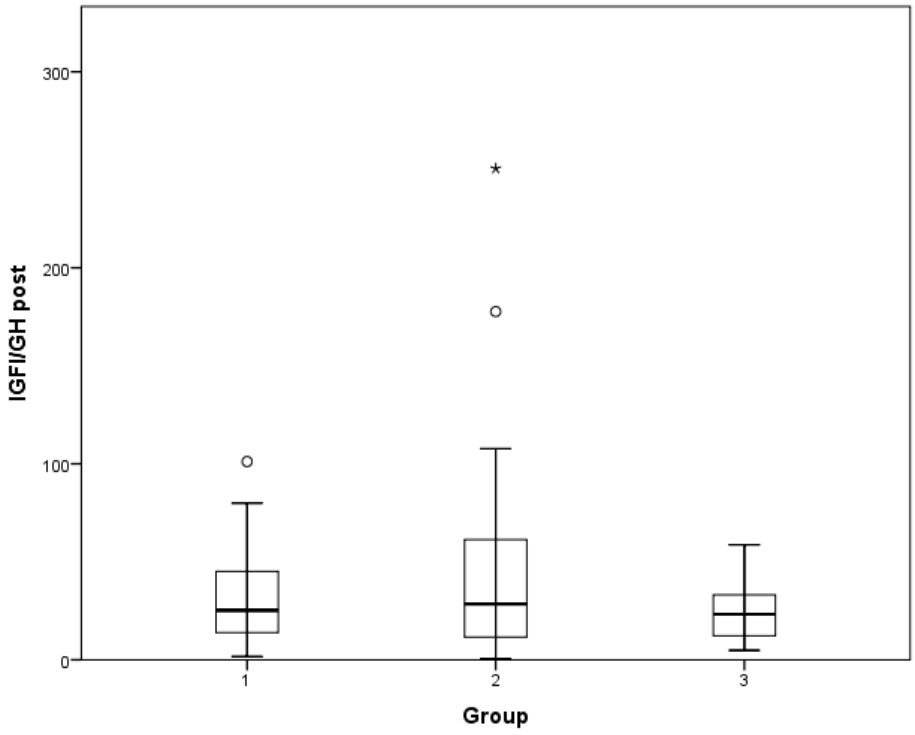


Figure 4.4 Box Plots to compare the three genotype groups for IGF-I/GH post primary treatment

This box plot represents the post treatment results for IGF-I/GH for the three genotype groups; group 1= fl/fl, group 2= fl/d3 and group 3= d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers”. Occasional outliers are shown as circles and asterix.

Comparison of the three genotype groups was performed using non-parametric testing; no statistically significant difference was detected between the genotype groups.



4.3.3.2 Diagnostic Data

Further analyses were performed of the subgroup of 44 patients for whom diagnostic data were available. GH and thus IGF-I/GH results were again skewed and non-parametric analyses were performed.

GH levels were median 39.2 (range 5.1-248.2) for fl/fl, 43.1 (6.8-527.6) for fl/d3 and 45.4 (10.6-110.3) for d3/d3 at diagnosis; there was no statistically significant difference between genotype groups (p=0.968) (table 4.2b, figure 4.3b). IGF-I %ULN levels were 301.7 (125-545) for fl/fl, 316.9(134-483.1) for fl/d3 and 293 (187.5-700.4) for d3/d3; there was no statistically significant difference between genotype groups (p=0.806) (table 4.2b, figure 4.3a). IGF-I/GH levels were 6.85 (1.27-39.7) for fl/fl, 3.27 (0.41-30.8) for fl/d3 and 6.35 (4.31-36.6) for d3/d3; again there was no statistically significant difference between genotype groups (p=0.395) (table 4.2b, figure 4.3c).

Table 4.3 Comparison of the three GHR genotypes for GH and IGF-I levels taken at diagnosis

This table summarises the GH and IGF-I results for the subgroup of 44 patients for whom diagnostic GH and IGF-I results were available; the top half details the diagnostic results and the bottom half of the table demonstrates the post primary treatment results for this subgroup of the cohort. The left half of the table demonstrates the numbers of patients and the male to female distribution within each genotype cohort; full length homozygote fl/fl, d3 heterozygote fl/d3 and d3 homozygote d3/d3.

The median and (range) for GH, IGF-I and GH/IGF-I are shown for each genotype group. The right hand side of the table shows the results for the comparison between the genotype groups for the above measures using Kruskall Wallis non-parametric analyses in view of the skewed data. Statistical significance is taken at p≤0.05.

Abbreviations: fl/fl: full length homozygotes; fl/d3: d3 heterozygotes; d3/d3: d3 homozygotes; IGF-I %ULN: % upper limit of normal reference range for IGF-I

	fl/fl	fl/d3	d3/d3	*p value*
Number (% Female)	24 (62.5)	14 (35.7)	7 (28.6)	
IGF-I %ULN	301.7 (125-545)	316.9 (134.0-483.1)	293.0 (187.5-700.4)	*0.806*
Mean GH (miu/l)	39.2 (5.1-248.2)	43.1 (6.8-527.6)	45.4 (10.6-110.3)	*0.968*
IGF-I/GH	6.85 (1.27-39.7)	3.27 (0.41-30.8)	6.35 (4.31-36.6)	*0.395*

Figure 4.5 Box Plot to demonstrate Diagnostic IGF-I%ULN Results for the three genotype groups

This box plot represents the results for IGF-I %ULN for the three genotype groups at diagnosis; group 1= fl/fl, group 2= fl/d3 and group 3= d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers”. Occasional outliers are shown as circles and asterix. Comparison of the three genotype groups was performed using non-parametric testing; no statistically significant difference was detected between the genotype groups.

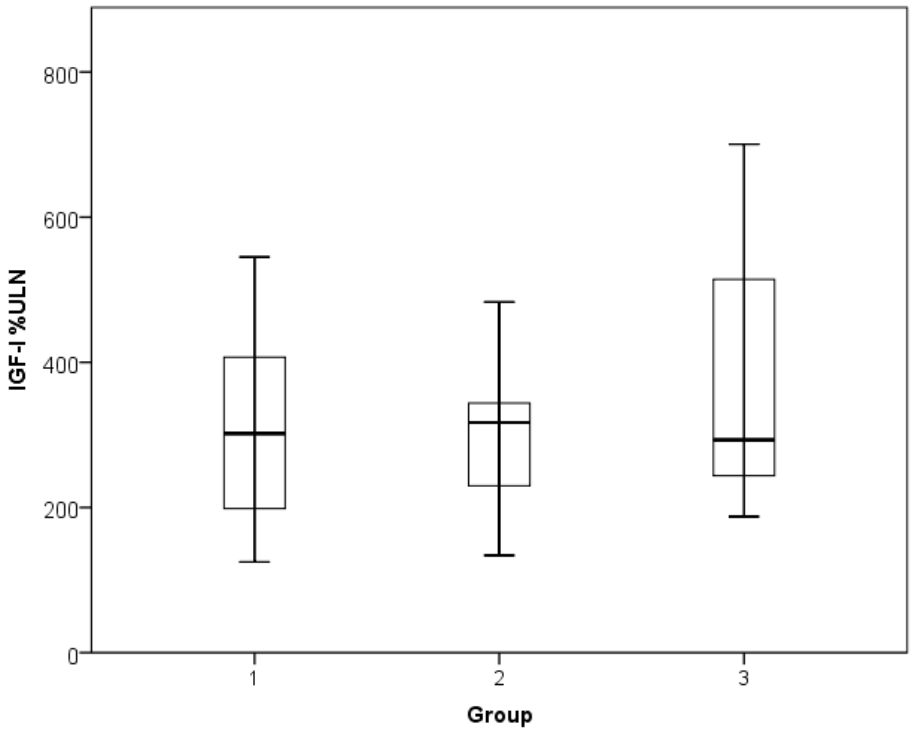


Figure 4.6 Box Plot to demonstrate Diagnostic GH Results for the three genotype groups

This box plot represents the results for the mean GH from a 5 point day curve for the three genotype groups at diagnosis; group 1= fl/fl, group 2= fl/d3 and group 3= d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers”. Occasional outliers are shown as circles and asterix.

Comparison of the three genotype groups was performed using non-parametric testing; no statistically significant difference was detected between the genotype groups.

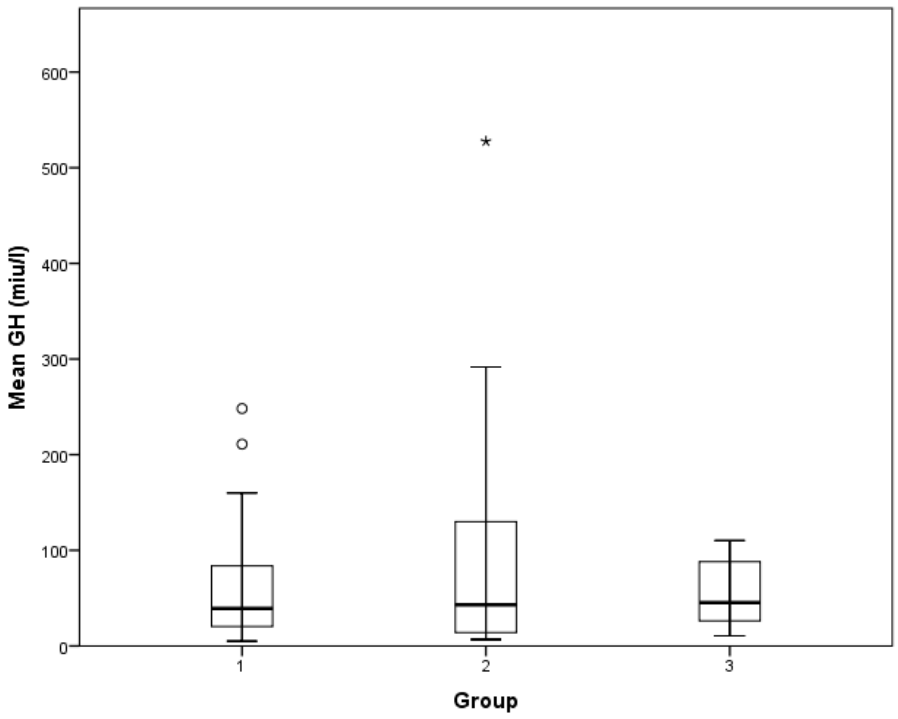
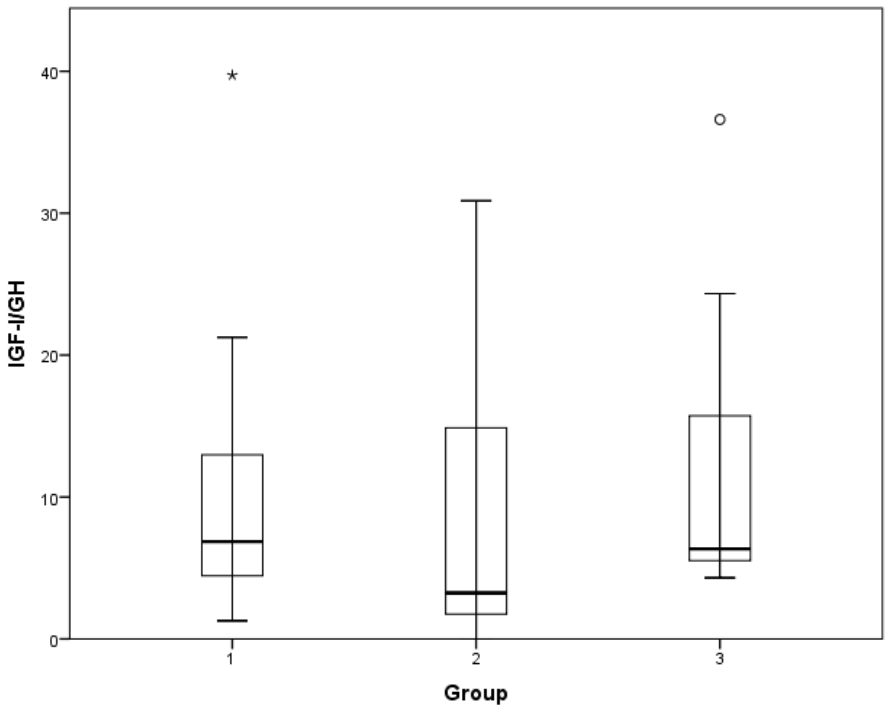


Figure 4.7 Box Plot to demonstrate Diagnostic IGF-I/GH Results for the three genotype groups

This box plot represents the results for IGF-I/GH for the three genotype groups at diagnosis; group 1= fl/fl, group 2= fl/d3 and group 3= d3/d3. The box shows the distribution from the 25th to 75 centile, the line within the box denotes the median with the range demonstrated by the “whiskers”. Occasional outliers are shown as circles and asterix.

Comparison of the three genotype groups was performed using non-parametric testing; no statistically significant difference was detected between the genotype groups.



4.3.4 Regression analyses of the effect of d3 homo and heterozygosity on log_e(IGF-I%ULN)

GH and IGF-I%ULN were converted to $log_{e(}$GH) and log_e(IGF-I%ULN) to minimise the impact of outliers; this yielded a linear relationship for both post treatment data (figure 4.4a) and diagnostic data (figure 4.4b). The slopes were greater for post treatment data with lower GH values, than for the pre-treatment values; this is consistent with individuals having a ceiling on IGF-I generation irrespective of further increases in GH.

4.3.4.1 Post Primary Treatment

Analysis of the post treatment data for the whole cohort confirmed a significant relationship between $log_{e(}$GH) and log_e(IGF-I%ULN) (figure 4.4a, table 4.3a). Neither d3 homo (d3/d3) or heterozygosity (fl/d3) were confirmed to have a significant effect on log_e(IGF-I%ULN) measured 3 months post primary treatment (p=0.708 for d3/d3, p=0.560 for fl/d3) (table 4.3a). As both demonstrated positive, albeit non-significant results, further analyses of fl/fl were performed; this confirmed a non-significant effect on log_e(IGF-I%ULN) (p=0.533) (table 4.3a).

28 of the 79 patients achieved biochemically normal GH and IGF-I levels post primary treatment; to ensure that these “normal” results have not masked the effect of the d3-GHR polymorphism on GH responsiveness, analyses were repeated excluding these patients from the analyses. Repeat regression of this subgroup confirmed that there was no statistically significant effect of either fl/d3 (p=0.172) or d3/d3 (p=0.752) (table 4.3b).

4.3.4.2 Diagnostic Data

Analysis of the subgroup for whom diagnostic data were available confirmed a linear relationship between $\log_{e(}$GH) and $\log_e$(IGF-I%ULN) (figure 4.4c) with a significant relationship between $\log_e$(GH) and $\log_e$(IGF-I%ULN); p=0.032 (table 4.3c). Possession of one or more d3 allele did not have a significant effect on $\log_e$(IGF-I%ULN); p=0.2 for fl/d3 and 0.801 for d3/d3 (table 4.3c).

Figure 4.8 Graph to demonstrate the relationship between loge(GH) and loge(IGF-I%ULN) measured at 3 months post primary treatment

This plot demonstrates the distribution of post primary treatment GH and IGF-I%ULN results for the cohort studied. A linear relationship is demonstrated between GH and IGF-I; $\log_e$(GH) and $\log_e$(IGF-I%ULN) were used to minimise the impact of outliers. Regression analyses confirmed a statistically significant relationship between $\log_e$(GH) and $\log_e$(IGF-I%ULN) (p=0.00).

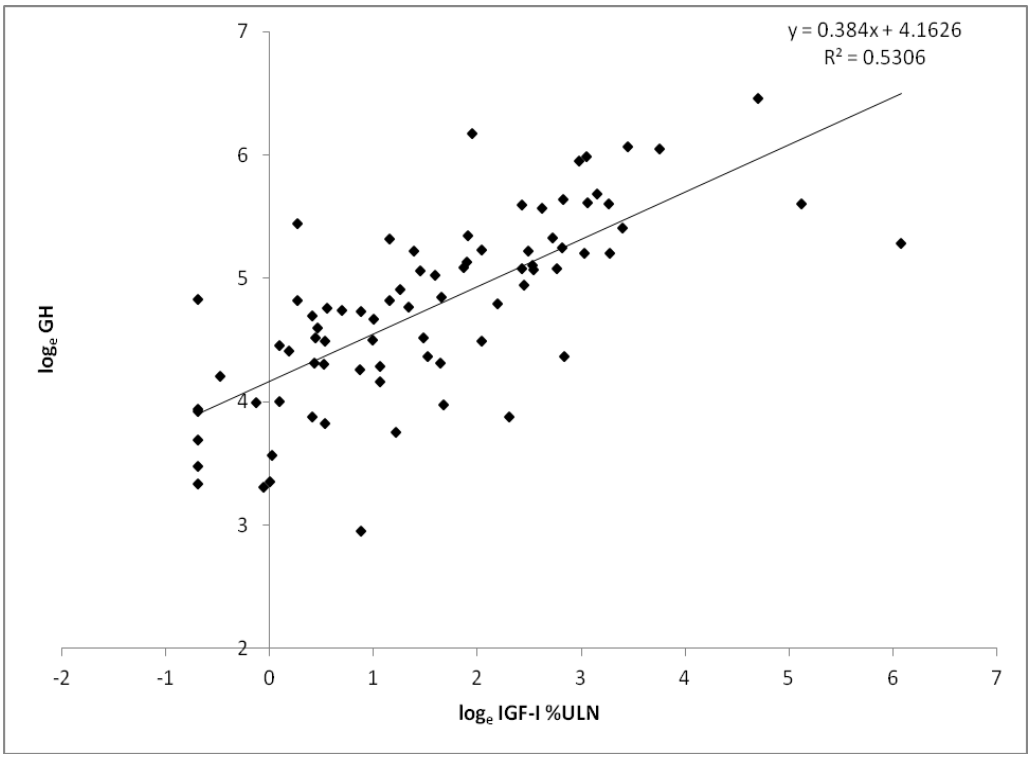


Table 4.4 Multiple Regression analyses investigating factors influencing GH responsiveness: Post Primary Treatment

The main question is the relationship between GH and IGF-I and how variations in GHR genotype may influence the IGF-I response. Regression analyses were performed to investigate the relationship between GH and IGF-I as demonstrated by $\log_e$(IGF-I %ULN) and $\log_e$(GH) and the effect of d3 homo (d3/d3) and heterozygosity (fl/d3) on IGF-I levels. This table demonstrates the results of these analyses in conjunction with analyses of the potential confounders, oestrogen and pituitary irradiation on IGF-I levels taken at a single, comparative time point of 3 months post primary treatment. Results for the whole cohort of 79 patients were used for these analyses. As both fl/d3 and d3/d3 yielded positive but non-significant results, further analysis of fl/fl was performed to investigate whether these results were different; this is shown at the bottom of the table and confirms a non-significant result.

Statistical significance is taken at ≤0.05.

	$\log_e$(IGF-I %ULN)	
	β-Coeff	p value
$\log_e$(GH)	0.746	**0.00**
fl/d3	0.049	0.560
d3/d3	0.032	0.708
Oestrogen	-0.47	0.643
Pituitary irradiation	-0.60	0.481
R squared	0.548	
fl/fl	-0.050	0.533

Figure 4.5 Graph of the relationship between loge(GH) and loge(IGF-I%ULN) measured at 3 months post primary treatment: non-cured subgroup

This plot demonstrates the distribution of post primary treatment GH and IGF-I%ULN results for the subgroup within the cohort who did not achieve a GH <5miu/l and an IGF-I within the normal age related reference range post primary treatment. Regression analyses of this subgroup confirmed a statistically significant relationship between $\log_e$(GH) and $\log_e$(IGF-I%ULN) (p=0.00) but d3-GHR genotypes had no effect on $\log_e$(IGF-I%ULN).

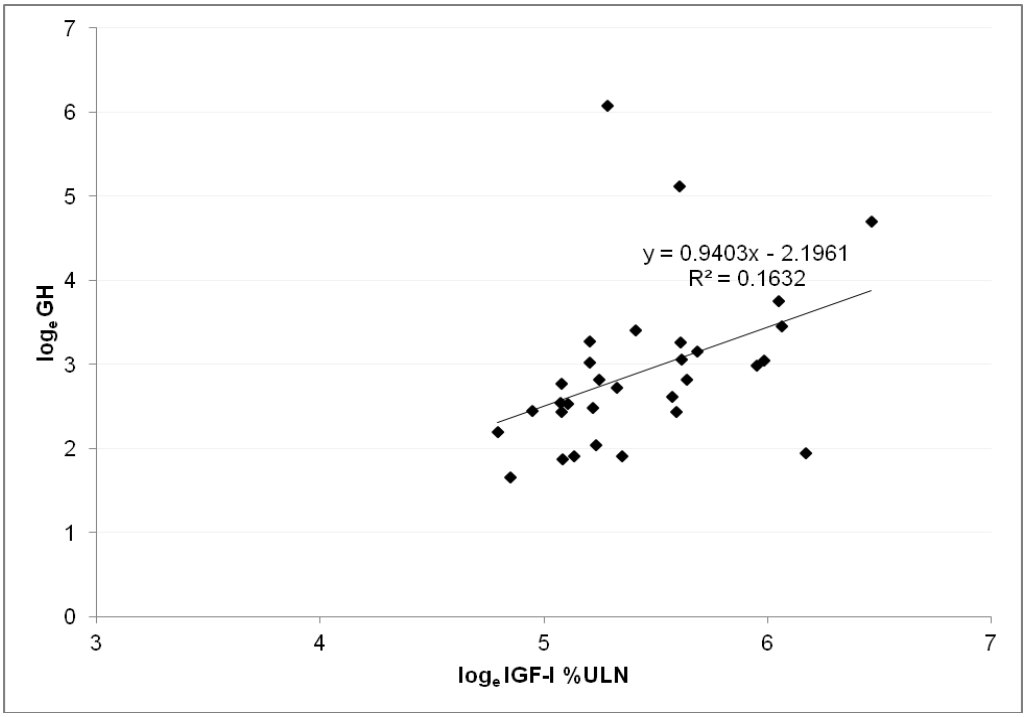


Table 4.6 Multiple Regression analyses: Post Primary Treatment Subgroup analysis

This table summarises the results of the regression analyses of the subgroup who failed to achieve a normal GH and IGF-I level post primary treatment; this was performed to ensure that "normal" GH and IGF-I results were not masking the effect of the d3-GHR polymorphism. Neither d3/d3 or fl/d3 genotypes were found to have a significant effect on IGF-I level (log_e IGF-I %ULN).

	log_e(IGF-I %ULN)	
	β-Coeff	p value
log_e(GH)	0.369	0.041
fl/d3	-0.251	0.172
d3/d3	0.057	0.752
Oestrogen	-0.219	0.320
Pituitary irradiation	0.152	0.400
R squared	0.362	

Figure 4.9 Graph to demonstrate the relationship between loge(GH) and loge(IGF-I%ULN) measured at diagnosis

This plot demonstrates the relationship between $\log_e$(GH) and $\log_e$(IGF-I%ULN) in the cohort for whom diagnostic data were available (n=44). A linear relationship between $\log_e$(GH) and $\log_e$(IGF-I%ULN) was confirmed and was statistically significant (p=0.032).

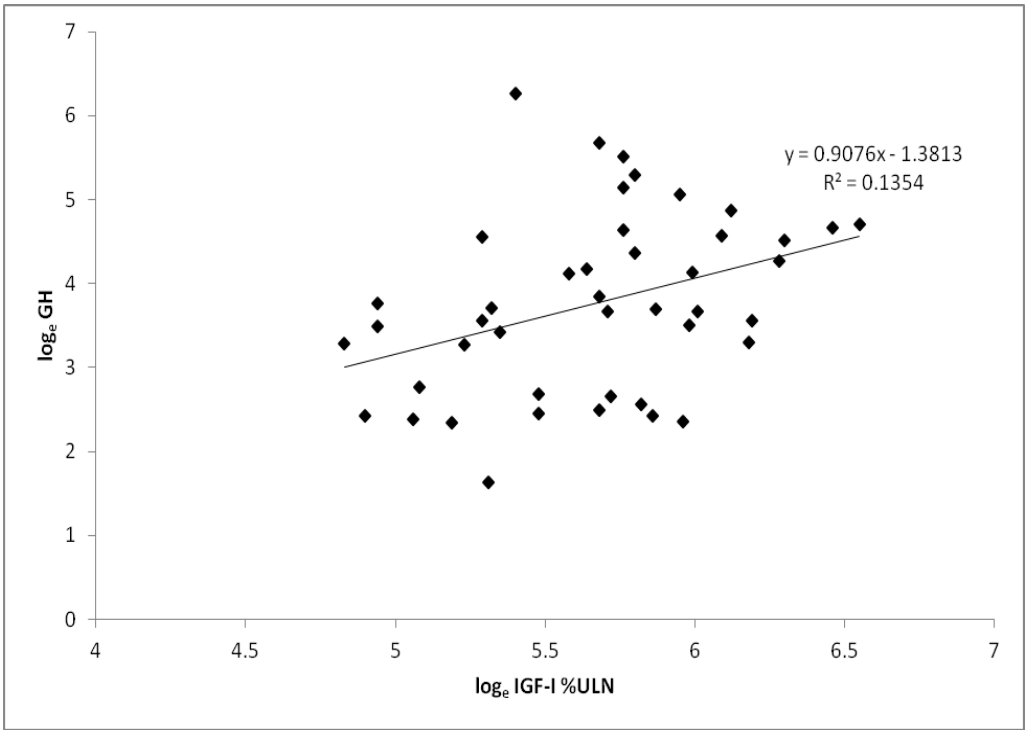


Table 4.7 Multiple Regression analyses investigating factors influencing GH responsiveness: Diagnostic data

This table summarises the results of the analyses of the subgroup for whom diagnostic data were available. A multiple regression model was developed to investigate the effect of d3-GHR genotype and other factors on IGF-I levels (log_e(IGF-I %ULN) used for analyses). A statistically significant effect of log_e(GH) is seen. Neither d3 homo or heterozygosity were found to have a significant effect on log_e(IGF-I%ULN) at diagnosis. The presence of oestrogen as a potential confounder was included in the analyses and confirmed to not have a significant effect on the diagnostic data.

	log_e(IGF-I %ULN)	
	β-Coeff	*p value*
log_e(GH)	0.239	**0.032**
fl/d3	-0.146	0.200
d3/d3	0.29	0.801
Oestrogen	-0.117	0.398
R squared	0.174	

4.4 Discussion

Discordance in the relationship between GH and IGF-I remains poorly understood. Data in children has largely demonstrated an augmented linear growth response in those in possession of one or more d3-GHR allele. Data in adult studies of GH deficiency and excess have yielded conflicting results(Mercado, DaVila et al. 1994, Schmid, Krayenbuehl et al. 2007, Barbosa, Palming et al. 2008, van der Klaauw, van der Straaten et al. 2008, Kamenicky, Dos Santos et al. 2009) and the question arises as to whether the d3-GHR polymorphism has a significant effect on GH response or whether the lack of an easily measureable and reproducible marker of GH activity in adults is masking its effect. The aim of this study was to investigate the effect of the d3-GHR polymorphism on GH responsiveness in a cohort of patients with acromegaly.

Summary of Results

GHR Genotyping

The genotyping of 79 patients with acromegaly from a single centre yielded similar results to those published previously. 50.6% of the population were full length homozygotes, 36.7% were d3 heterozygotes and 12.6% were d3 homozygotes. As previously described, misclassification of d3 heterozygotes as homozygotes was evident in 2 out of 12 subjects; a second PCR was necessary to confirm these results. A 20% misclassification rate was previously demonstrated (Carrascosa, Esteban et al. 2006) and this may be in part responsible for the variability in results published by previous authors.

Analysis of GH responsiveness between genotype groups

The study hypothesis was that possession of one or more d3 allele enhances GH responsiveness as shown by an augmented IGF-I response to a given level of GH in patients with acromegaly. A cohort of 79 patients was studied; these demonstrated a wide range of ages, disease severity and duration since diagnosis from weeks to decades. Paired GH and IGF-I results were used for analysis; due to the wide variability in the time of diagnoses and the lack of IGF-I assay pre-1994, diagnostic data were not available for the whole cohort. Instead a time point of three months post primary treatment was used to allow standardisation of results for comparison with a subgroup analysis of those patients with diagnostic results; using this time point, data were available for analysis in all patients. Response to treatment or the effect of different treatment types was not analysed as the hypothesis specifically relates to the relationship between GH and IGF-I; the only treatment that would have an effect on this relationship is pegvisomant which was not used as primary therapy in any of the cohort studied.

Non parametric tests were used in the comparison of GH, IGF-I (reported as % upper limit of normal) and IGF-I/GH for the three genotype groups fl/fl, fl/d3 and d3/d3, in view of the skewed data. No significant difference was detected between the three genotypes for either GH, IGF-I or IGF-I/GH in the analyses of post treatment results and diagnostic data.

More detailed analyses of the relationship between GH and IGF-I were performed using regression analyses. As expected, the plot of $\log_e$ GH and $\log_e$ IGF-I %ULN yielded a linear relationship between GH and IGF-I. A steeper slope was evident with the post treatment data in relation to the diagnostic data; this suggests that above a certain level of GH there may be saturation of the GHR and consequently further IGF-I may not be generated.

Multiple regression analyses confirmed the positive, linear relationship between GH and IGF-I; analyses of d3 homo- and heterozygotes however failed to demonstrate a significant effect on IGF-I level. As both groups showed non-significant but positive effects in the post treatment cohort, further analyses of the fl/fl group were performed; this excluded a significant negative effect. In order to ensure that the inclusion of patients with “normal” GH and IGF-I levels in the post treatment analysis were not influencing results, repeat analyses of the subgroup of patients with persistently abnormal GH and IGF-I results post treatment. This repeat analysis did not demonstrate a statistically significant effect of the d3-GHR polymorphism on GH responsiveness in the post treatment group. Further analyses were also performed of the 44 patients for whom diagnostic GH and IGF-I results were available; again, no statistically significant effect of d3 homo or heterozygosity on GH responsiveness was observed.

In summary, despite robust statistical analyses of a large cohort of patients with acromegaly, there was no evidence of an enhanced IGF-I response to GH in either the d3-GHR homozygous or d3-GHR heterozygous groups.

Critique of Work

The strengths of this study are in the methodology. Detailed, robust statistical analyses were performed with input from an expert statistician to ensure validity of the results and conclusions. In order to avoid masking any subtle effects of the polymorphism, d3 homo and heterozygosity were analysed separately. Furthermore, a second PCR was performed for all labelled d3 homozygotes to avoid the potential for misclassification as reported by Carrascosa et al (Carrascosa, Esteban et al. 2006); consequently 2 out of 12 patients were reclassified as d3

heterozygotes. Despite detailed analyses, there was no evidence of an augmented IGF-I response to GH in those possessing one or more d3 allele; as such our original hypothesis was not supported by these data.

This study was however limited by the availability of data; although the original cohort was 79 patients, diagnostic IGF-I levels were only available for 44 patients. Although in theory, paired GH and IGF-I levels could be analysed from any time point, analysing raw data from diagnosis would be preferable in order to standardise results for comparison.

Analysis of the whole cohort for post treatment results showed no statistically significant effect of the d3-GHR polymorphism on IGF-I generation for given GH stimulus. It should however be noted that 28 of the 79 patients achieved GH levels of <5miu/l and an IGF-I within normal reference range after the primary treatment. This raises the question of whether any change in GH responsiveness due to the d3-GHR polymorphism would be present or detectable when GH levels are normal. Further analyses excluding those who achieved “normal” GH levels within this cohort did not detect any difference in IGF-I response in the d3-GHR genotype groups; it would however be preferable to have a larger cohort with abnormal GH and IGF-I results to maximise statistical power. In contrast to the study presented within this thesis from patients with GHD, it proved more problematic to recruit patients with acromegaly into this study. The 79 patients recruited represent approximately 20% of the department’s acromegaly caseload. The most likely explanation for the discrepancy in recruitment rates relates to the close clinical relationship with GHD patients and the department’s hypopituitary/GH clinical nurse specialist, whose help in recruiting GHD patients was invaluable.

Consideration of these data in the context of the Published Literature

This data has added to the confusion over whether the d3-GHR polymorphism has a significant role in determining an individual's response to GH. Several studies in a range of adult and children patient cohorts have yielded conflicting results; either demonstrating a pronounced augmentation of GH response or negative or equivocal GH responses.

In adults with acromegaly, four other studies have preceded this one and even in spite of the predominantly positive results, the inconsistent effect of d3-GHR is apparent and does raise the question of how clinically relevant this polymorphism is. Mercado et al demonstrated no significant difference in IGF-I levels at baseline but higher IGF-I levels in the combined d3-GHR group post treatment and a higher prevalence of diabetes(Mercado, Gonzalez et al. 2008). Schmid et al demonstrated higher serum GH levels at diagnosis in the fl/fl group compared to the combined d3 group with comparable IGF-I levels, thereby suggesting more IGF-I is generated for a given level of GH, however no association with clinical end points such as diabetes was detected(Schmid, Krayenbuehl et al. 2007). Bianchi et al also demonstrated a difference only after treatment (surgery or SA) but without any detectable difference at diagnosis(Bianchi, Giustina et al. 2009). A Dutch study of 80 patients (Wassenaar, Dekkers et al. 2009) demonstrated an increase in the irreversible complications of acromegaly (osteoarthritis and colonic polyps) in the combined d3 group but in the absence of a detectable difference in IGF-I levels between d3+ and - groups. Since the majority of the effects of GH are mediated through IGF-I and the effect of the d3-GHR is purported to be to enhance the IGF-I response, it is unclear how or why this polymorphism has resulted in such a significant increase in complications. Rather than demonstrating a clear effect of the d3-GHR in enhancing GH response, it suggests that there must be another factor

that is augmenting the response to IGF-I, such as variability in the IGF-I receptor. Most recently, a study of 105 acromegalic patients failed to show any difference in GH or IGF-I levels between genotype groups and furthermore failed to show any difference in correlation (Kamenicky, Dos Santos et al. 2009). Cumulatively the lack of a conclusive, significant result from these studies does suggest that there must be other more relevant GH responsiveness factors other than the d3-GHR polymorphism.

Future Work

The absence of a conclusive result from this or other published studies suggests that the d3-GHR polymorphism is not a major factor in determining an individual's response to GH. Whilst it may be that the lack of an easily measurable and reproducible marker of GH activity in adults is influencing results, other potential GH responsiveness factors must now be given more consideration.

Chapter 5

Study to determine the optimum serum IGF-I range in patients with acromegaly treated with Pegvisomant

5.1 Introduction

Pegvisomant, a GH receptor antagonist, is a highly effective treatment for acromegaly with clinical and biochemical response rates of up to 97% reported (van der Lely, Hutson et al. 2001). Monitoring of treatment can be difficult; the similarity of pegvisomant to GH dictates that, for many assays, serum GH levels cannot be used to guide treatment (Veldhuis, Bidlingmaier et al. 2001) leaving serum IGF-I as the sole marker of disease activity. There are a number of problems with this (unavoidable) approach. First, IGF-I has a wide normal reference range and, without knowledge of an individual patient's GH/IGF-I physiology prior to the development of acromegaly, it is not clear where, within that reference range, GH activity is at its most "normal." Second, variations in IGF-I assay quality are well recognised (Pokrajac, Wark et al. 2007) and many do not take account of the known gender difference in IGF-I generation for a given excess GH stimulus (Parkinson, Ryder et al. 2001). Third, as discussed earlier in this thesis, there is a known 30% discordance rate between GH and IGF-I for unknown reasons. Characteristic physiological and metabolic changes occur in relation to both GH deficiency and excess. For example, active acromegaly is associated with increased lean body mass, decreased fat mass, redued C-reactive protein (CRP) levels, sodium and water retention and accelerated lipolysis. In contrast, untreated severe adult-onset GHD is characterised by decreased muscle mass, visceral adiposity, elevated CRP, a reduction in totally body sodium and fasting hyperlipidaemia. The aim of this study, therefore, was to document changes in GH-dependent metabolic parameters with pegvisomant treatment, in order to determine where within the reference range of IGF-I 'optimum' biochemical control of acromegaly lies (thereby acting as a guide for pegvisomant treatment) and to explore whether it is possible that a state of

'functional/pharmacological' GHD may be induced by excessive pegvisomant dosing in this condition.

5.1.1 Aim of this section

- To induce a metabolic syndrome similar to GH deficiency with the use of supra-physiological doses of pegvisomant to allow the identification of an 'optimum' range of serum IGF-I to guide pegvisomant dosing.

5.2 Study Design

This clinical trial was approved by a central ethics committee (Reference 07/H0703/126) and by the Medicines Healthcare Regulatory Agency (EudraCT No: 2007-003741-33).

A cohort of 10 patients currently receiving pegvisomant treatment for active acromegaly was prospectively recruited; 1 patient subsequently withdrew from the study due to personal reasons. All patients were already taking pegvisomant for acromegaly that was refractory to other treatment (i.e previous non curative surgery, radiotherapy or failure to achieve remission with somatostatin analogues). The following inclusion and exclusion criteria were applied:

5.2.1 Inclusion criteria:

- Active acromegaly on pegvisomant monotherapy at a stable dose with a normal age-adjusted serum IGF-I for at least 3 months
- Over 18 years of age
- Willing to provide informed consent

5.2.2 Exclusion criteria:

- Unwilling or unable to provide informed consent
- Other conditions known to alter IGF-I levels (severe hepatic disease, severe renal disease, malnutrition, ethanol and drug abuse)
- Abnormal liver enzymes
- Pregnancy/lactation

All had achieved biochemical control of acromegaly, as evidenced by a normal serum IGF-I level on their maintenance dose of pegvisomant. All study participants provided informed, written consent.

5.2.3 Clinical Assessment

The following markers of GH activity were taken at baseline whilst on their maintenance dose of pegvisomant:

- Body Composition: DXA % Body fat and waist: hip ratio
- Glycaemic control and insulin resistance: fasting insulin and glucose and **HOMA2 IR analysis**

- Cardiovascular risk: Lipoprotein (a), fibrinogen, CRP
- Quality of life: AcroQoL, EuroQoL, AGHDA questionnaires (Appendix)

All patients then followed a dose titration of pegvisomant, with monitoring of serum IGF-I levels on a 2 weekly basis, aiming for just below the lower limit of the age adjusted reference range. Once target IGF-I was achieved, the dose of pegvisomant was continued for a 12 week period and the above measurements were reassessed at the end of the trial period.

5.2.4 Statistical model

The proposed statistical model to identify the optimal IGF-I range from the distribution of results of the physiological markers of GH activity was as follows: perform a regression of IGF-I against physiological marker to form regression line IGF-1 = $\alpha + \beta \times M$, where α is the value of IGF-1 at M=0 and β is the slope of the line. The range of IGF-I values that correspond to the 'safe' range of the metabolic marker are: $\alpha + \beta \times m_1$ and $\alpha + \beta \times m_2$. The range can then be narrowed by adding and subtracting 1.96 times the within-person standard deviation of paired measurements of IGF-1from the same sample from the lower and upper intervals of the range (figure 5.1). The proposed model requires a full set of data of IGF-I levels with paired physiological markers taken at baseline and with a subnormal IGF-I induced by over treatment with pegvisomant.

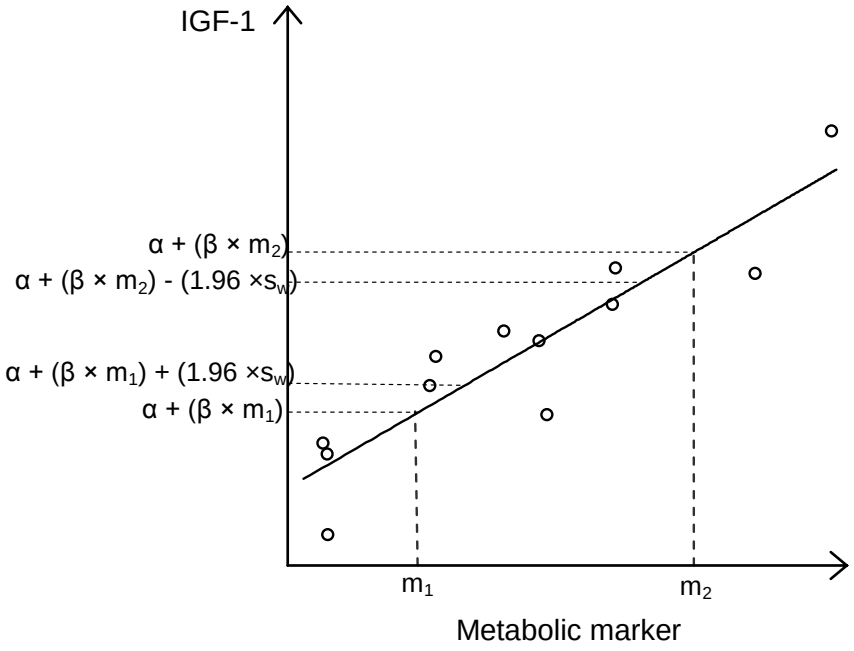
In order to investigate for differences in the physiological markers between baseline and after 12 weeks at an increased dose of pegvisomant, non-parametric tests were performed due to the small sample size. Results are reported as median (range) due to the small sample size. Statistical significance was accepted at a p value

<0.05. Analysis was performed using SPSS (version 11.01; SPSS Inc, Chicago, IL) for Windows XP (Microsoft Corp).

Figure 5.1 Statistical model to identify the range of IGF-I which corresponds to normalisation of physiological markers of GH activity

This graph demonstrates the proposed model for calculation of the optimal range of IGF-I in pegvisomant treatment. A regression line of IGF-I against physiological marker; IGF-1 = $\alpha + \beta \times M$, where α is the value of IGF-1 at M=0 and β is the slope of the line. The range of IGF-I values that correspond to the 'safe' range of the physiological marker are: $\alpha + \beta \times m_1$ and $\alpha + \beta \times m_2$. To further narrow the 'safe' range, add and subtract 1.96 times the within-person standard deviation of paired measurements of IGF-1from the same sample from the lower and upper intervals of the range.

Figure 5.1 Statistical model to identify the range of IGF-I which corresponds to normalisation of physiological markers of GH activity



5.3 Pegvisomant Dose Titration

All patients followed the dosing regimen shown below with the aim of achieving an IGF-I just below the normal reference range. The section in italics was included as an amendment to the original protocol to allow an increase of the maximum dose to 50mg od; this is the maximum daily dose used in the original pegvisomant publications. Response to an increased pegvisomant dose was assessed by IGF-I measured two weeks post increase and subsequent changes were made as per protocol. Weekly doses were calculated as an equivalent daily dose (weekly dose divided by 7) and the above protocol was followed accordingly. Any increases in dose were administered as a single weekly dose, for example an additional dose of 10mg per day was administered as additional 70mg per week in addition to the usual maintenance dose.

Table 5.1.1 Pegvisomant Dosing regime

This table summarises the pegvisomant dosing schedule used in this study. The target for treatment was a serum IGF-I level below the normal reference range. IGF-I levels were rechecked two weeks after an increase in dose and further increases made as necessary. The doses in italics were an amendment to the original protocol in view of the difficulty experienced in achieving target IGF-I.

Table 5.1 Pegvisomant Dosing regime

Dose of pegvisomant		Increase by	Dose
5 mg od		5 mg	10mg
10mg	IGF-I below median	5mg	15 mg od
	IGF-I above median	10mg	20 mg od
15 mg od	IGF-I below median	5mg	20 mg od
	IGF-I above median	10mg	25 mg od
20 mg od	IGF-I below median	10mg	30 mg od
	IGF-I above median	15mg	35 mg od
25 mg od	IGF-I below median	10mg	35 mg od
	IGF-I above median	15mg	40 mg od
30 mg od	IGF-I below median	10mg	40 mg od
	IGF-I above median	15mg	45 mg od
35mg od	*IGF-I below median*	*10mg*	*45mg*
	IGF-I above median	*15mg*	*50mg*
40mg od	*IGF-I below median*	*10mg*	*50mg*
	IGF-I above median	*10mg*	*50mg*

5.4 Results

5.4.1 Patient Cohort

10 patients with active acromegaly treated with pegvisomant for acromegaly and refractory to standard medical treatments (in accordance with clinical guidelines) were originally recruited. The cohort consisted of five male and five female patients aged between 32 and 74 years of age; one female patient subsequently withdrew from the study for personal reasons. All nine of the cohort had received prior pituitary surgery and/or external pituitary irradiation and/or medical therapy (Table 5.2). All had been treated with a stable dose of pegvisomant for a minimum of 3 months and had a normal age-adjusted serum IGF-I level prior to commencement of the study.

Table 5.1.2 Cohort Details

This table summarises the clinical details for the study cohort including diagnostic clinical and biochemical data and previous treatment received for acromegaly. The IGF-I levels taken prior to commencing pegvisomant as part of their clinical care is also stated; this demonstrates the severity of their GH excess despite previous treatment with surgery, radiotherapy and medical treatments.

IGF-I levels are reported as % upper limit of normal; this allows direct comparison of results measured on different assays.

Key: Diag: diagnosis, TSS: trans-sphenoidal surgery, RT: external beam radiotherapy, RS: radiosurgery, DA: dopamine agonists, SA: somatostatin analogs, %ULN: IGF-I level reported as % upper limit of normal reference range, * data not available, IGF-I Pre Pegv: IGF-I level taken prior to commencing pegvisomant.

Table 5.2 Cohort Details

Pt	Age M/F	IGF-I Diag ng/ml (%ULN)	Mean GH Diag miu/l	Tumour size at diagnosis	Previous Treatment					Pre-Pegv IGF-I ng/ml (%ULN)
					TSS	RT	RS	DA	SA	
1	74 F	*not available	200	"enlarged fossa"	N	N	Y	Y	Y	452 (226%)
2	48 M	86.8 (139.5%)	43.1	Macro	Y x2	Y	Y	Y	Y	478 (189.7%)
3	59 F	120 (187.5%)	26.3	Macro	Y	Y	N	Y	Y	416 (184.8%)
4	64 M	1022 (454.2%)	130.4	macro	Y	Y	N	N	Y	703 (312.4%)
5	56 M	1205 (535.5%)	71.4	macro	Y	Y	Fail	Y	Y	556 (247%)
6	69 M	* not available	106.3	Macro	Y	Y	Y	Y	Y	365 on SA (182.5%)
7	48 F	* not available	88.0	Macro	Y	Y	N	Y	Y	407 (161.5%)
8	46 M	988 (392%)	199	Macro	Y	N	N	Y	Y	678 (269%)
9	32 F	1001 (280.4%)	197	Macro	Y	Y	N	Y	Y	732 (204%)

5.4.2 Response to Pegvisomant dose titration

Pegvisomant doses were increased as detailed in section 5.3 with the aim of achieving a serum IGF-I just below the normal age-adjusted reference range. The summary of the dosing schedule and IGF-I levels for all nine subjects are shown in table 5.3. IGF-I levels were converted to standard deviation scores using *N – mean / SD* using age and gender related normative data for the assay used; these data are shown in table 5.4. At baseline IGF-I SDS were median 2.08 (range -0.77 to 3.55) and on the maximum trial dose IGF-I SDS were median -1.43 (range -3.09 to 0.20) (Figure 5.2). One of the cohort had a high IGF-I SDS of 3.55; at the time of recruitment he satisfied inclusion criteria with a stable IGF-I level but was unexpectedly found to have a high IGF-I at his initial visit. In view of the limited number of patients on pegvisomant, he was included in the study.

There is no prior intellectual knowledge regarding the use of supra-physiological doses of pegvisomant; all previous clinical trials used clinically effective doses only aiming for normalisation of IGF-I rather than mild GH deficiency. Although reductions in IGF-I were seen in all patients (figure 5.2) with a decrease in IGF-I of median 54.7% (range 26.6-66.4%) (Table 5.3), only three patients achieved a target IGF-I of below the age and gender defined normal reference range (table 5.3). Of the six patients who failed to achieve the target IGF-I, four were on the maximum dose of pegvisomant (50mg). Due to a limitation of time caused by a prolonged titration phase, two patients were unable to increase to the maximum 50mg and completed the study on 40mg per day. The three patients who achieved a sub-normal IGF-I were all female; two were post menopausal and thus oestrogen deficient.

The inability to achieve target IGF-I in the whole cohort prevented completion of the full statistical analysis as per the original protocol; from the data available it was not

possible to determine the optimal range of IGF-I for monitoring of pegvisomant using the proposed statistical model. The data does however provide interesting information regarding the unexpected difficulty in inducing a subnormal serum IGF-I even with very high doses of pegvisomant.

Table 5.1.3 Summary of IGF-I levels at baseline and following increased doses of pegvisomant

This table summarises the IGF-I levels taken at baseline on the usual maintenance pegvisomant dose and subsequent changes to IGF-I (numbered 1 to 5) in response to the increased pegvisomant doses (numbered 1 to 5) for the study cohort of 9 subjects. Doses of pegvisomant were increased aiming for a target IGF-I level of below the lower limit of age and gender defined reference range as shown in italics.

Dose of pegvisomant is reported as mg per day (mg per week stated in brackets for those patients on a weekly dose). A maximum dose of pegvisomant of 50mg per day was stipulated based on data from previous clinical trials. The lowest IGF-I level achieved is shown in bold; six out of the nine patients failed to achieve target IGF-I despite the use of high dose pegvisomant.

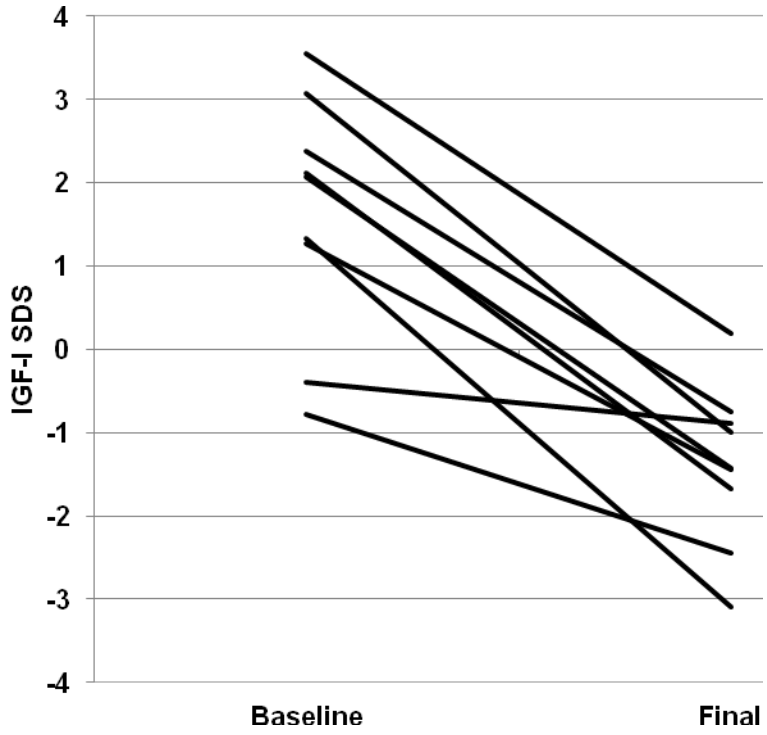
Table 5.3 Summary of IGF-I levels at baseline and following increased doses of pegvisomant

Pt	Baseline dose mg/day (mg/wk)	Baseline IGF-I	*Target IGF-I*	Dose (1)	IGF-I (1)	Dose (2)	IGF-I (2)	Dose (3)	IGF-I (3)	Dose (4)	IGF-I (4)	Dose (5)	IGF-I (5)	Trial dose	Weight kg	Target IGF-I achieved
1	10	90	*69*	15	140	20	115	25	102	30	87	35	**66**	35	59	Yes
2	15	250	*94*	15	214	25	147	30	147	35	118	40	**105**	40	92	No
3	10	182	*81*	20	104	25	**64**							25	65	Yes
4	8.6 (60)	250	*81*	15 (105)	174	25 (175)	123	35 (245)	106	40 (280)	**91**			40	101	No
5	20	285	*81*	35	241	50	**158**							50	105	No
6	20	223	*69*	35	149	50	**123**							50	95	No
7	10	130	*94*	15	104	20	**68**							20	68	Yes
8	14.3 (100)	248	*94*	20 (140)	227	30 (210)	228	40 (280)	175	50 (350)	**95**			50	86	No
9	30	243	*109*	45	188	50	**129**							50	82	No

Figure 5.2 Difference in IGF-I levels between baseline and trial end

This graph demonstrates the fall in serum IGF-I in response to the increased dose of pegvisomant. IGF-I levels are reported as standard deviation scores; -2 to +2 are considered normal. Each line represents an individual patient's IGF-I results at baseline and on the trial dose of pegvisomant. The aim was to increase the dose to lower the IGF-I level to below the normal reference range; although all IGF-I levels decreased in response to the increased dose only three patients achieved a level below the normal reference range.

Figure 5.2 Difference in IGF-I levels between Baseline and Trial end



5.4.3 Change in IGF-I

Although the majority of patients failed to achieve target IGF-I, all subjects experienced a reduction in IGF-I in response to the increased dose of between 26.7 and 66.4%. Figure 5.3 demonstrates the plot of change in IGF-I against change in pegvisomant dose for the cohort; it is apparent that although there appears to be a linear relationship, there are a number of outliers which suggests that an individual patient's biochemical response to pegvisomant is multi-factorial. To investigate this further, a regression analysis was performed to investigate the individual and cumulative effects of pegvisomant dose, gender and body weight on ΔIGF-I. Results are shown in table 5.4; none of the factors were found to have a statistically significant effect on ΔIGF-I.

Figure 5.3 Plot of ΔIGF-I against ΔPegvisomant Dose

This graph represents the plot of ΔIGF-I against ΔPegvisomant Dose for the cohort studied. Only three out of the nine patients studied achieved a target sub-normal IGF-I which suggests there is some variability in response to pegvisomant. This variability is further demonstrated in this plot; although the relationship of ΔIGF-I against ΔPegvisomant Dose is mostly linear, a number of outliers are present.

Figure 5.3 Plot of ΔIGF-I against ΔPegvisomant Dose

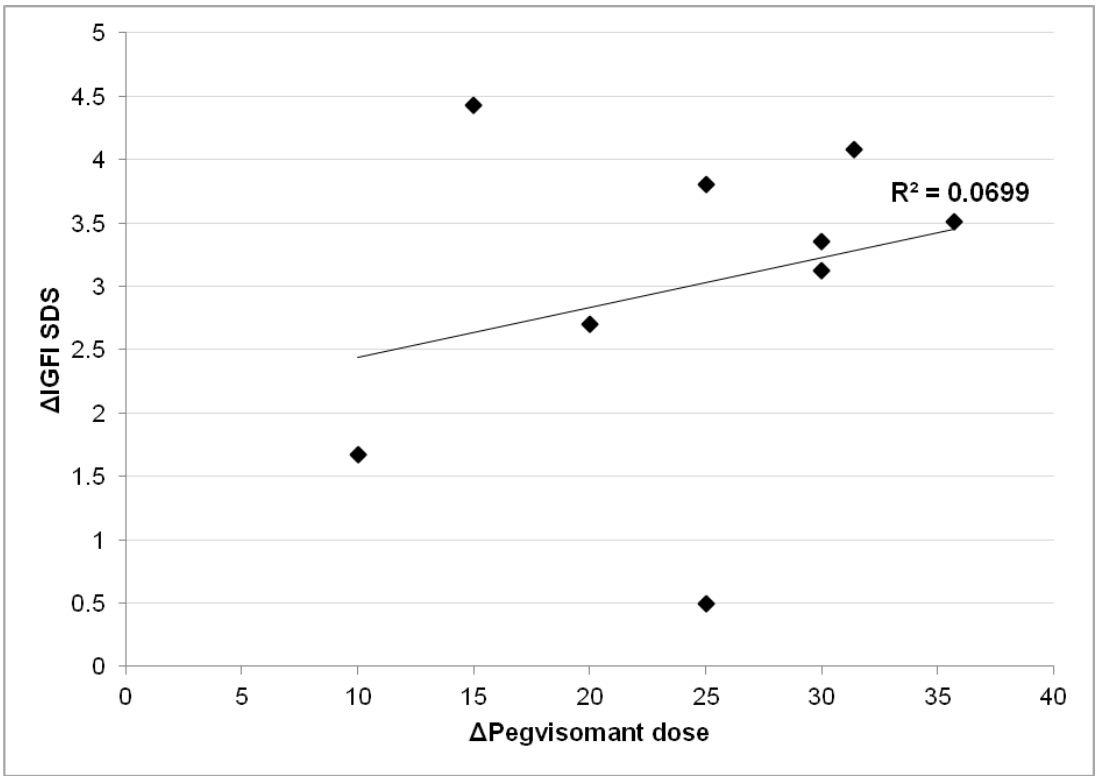


Table 5.1.4 Factors affecting IGF-I response to Increased Dose Pegvisomant

A linear regression model of ΔIGF-I SDS against change in dose, body weight, gender and maintenance pegvisomant dose was performed to investigate the individual and cumulative effects on ΔIGF-I SDS; none were found to have a statistically significant effect on ΔIGF-I.

	ΔIGF-I	
	β-Coeff	*p value*
Change in Pegvisomant Dose	-0.453	*0.561*
Maintenance Pegvisomant Dose	-0.81	*0.902*
Gender	-0.576	*0.680*
Body weight	0.377	*0.749*
R Squared	0.388	

5.4.4 Effect of Pegvisomant on Physiological Markers of GH activity

Characteristic physiological changes are expected in relation to GH activity levels; the most directly measurable relate to changes in body composition, insulin resistance, markers of cardiovascular risk and quality of life. These were assessed at baseline on the patient's maintenance pegvisomant dose and at the end of the trial period on the increased dose.

Fasting plasma glucose at baseline was median 5.0mmol/l (range 4.5-6.5) and at trial end median 4.9mmol/l (3.9-7.5); no statistically significant difference was detected (p= 0.373) using non parametric tests in view of the small sample size (Table 5.5). Repeat testing with removal of the outlier (figure 5.4a) confirmed no statistically significant difference (p=0.091). Fasting insulin levels were median 7.5miu/l (range 2.0-26.0) at baseline and 4.2miu/l (2.0-9.0) at the end of the trial; again no statistically significant difference detected (p=0.069) (figure 5.4b). There was also no significant difference in HOMA2-IR Insulin resistance scores; median 1.1 (0.4-3.5) at baseline and median 0.6 (0.4-1.3) on increased dose pegvisomant (p=0.075) (figure 5.4c).

Assessment of body composition revealed a significant difference in percentage body fat as measured by DXA; median 25.4% (19.4-36.5) at baseline and 26.5% (18.6.0-37.6) final result, p=0.036 (figure 5.5a). No difference was detected with waist:hip ratio however; median 0.87 (0.83-1.03) at baseline compared to 0.90 (0.83-0.98) on high dose pegvisomant (p=0.528) (figure 5.5b). Cardiovascular risk factors were assessed; a significant response to pegvisomant was seen with fibrinogen, baseline median 3.13g/l (2.31-3.92) compared to 2.86g/l (1.99-3.45) at trial end (p=0.012) (figure 5.6b). There was however no detectable difference with lipoprotein A; median 135mg/l (24-806) at baseline and 82mg/l (24-679) at trial end (p=0.345) (figure 5.6a).

No statistically significant differences were detected in the quality of life assessments. The Adult Growth Hormone Deficiency Assessment (AGHDA) score was median 3 (range 0-23) at baseline and median 5 (2 to 23) at trial end; this was not statistically significant p=0.102 (figure 5.7a).

AcroQol; a specific acromegaly related questionnaire of physical and psychological wellbeing, also showed no statistically significant difference between baseline (median 86.4, range 29.5 to 109.1) and trial end (median 80.7, range 15.9-98.9) p=0.173 (figure 5.7b). The visual analog scale of EuroQoL, a non disease specific QoL questionnaire, also demonstrated no statistically significant difference; median 75 (50-95) at baseline and median 80(50-95) on high dose pegvisomant, p=0.344 (table 5.5, figure 5.7c).

The aim of the study was to induce 'functional/pharmacological' GHD; due to the inability to achieve target IGF-I in the majority of the cohort, it was not possible to perform the statistical analyses initially planned due to the overlap of results. Figure 5.8 uses fasting plasma glucose as an example to demonstrate this overlap of results; it is not possible to calculate the difference between mean + and - 1.96SD for the two sets of results. Similar plots were evident for all markers of GH activity assessed.

Table 5.1.5 Response of Physiological Markers of GH to Increased Pegvisomant Dose

This table summarises the results taken at baseline on maintenance pegvisomant dose in comparison to measurements taken after 3 months of overtreatment, with a serum IGF-I below or close to the lower limit of the normal reference range. Measurements were made of characteristic markers of GH activity; body composition, insulin resistance, cardiovascular risk and quality of life scores. Non-parametric tests (Wilcoxon signed rank) were performed in view of the small sample size; significance is taken at $p \leq 0.05$.

	Baseline Median (Range)	Final Median (Range)	*p value*
Fasting Plasma Glucose	5.0 (4.5-6.5)	4.9 (3.9-7.9)	*0.373*
Fasting Insulin	7.5 (2.0-26.0)	4.2 (2.0-9.0)	*0.069*
HOMA2-IR	1.1 (0.4-3.5)	0.6 (0.4-1.3)	*0.075*
Fibrinogen	3.13 (2.31-3.92)	2.86 (1.99-3.45)	*0.012*
Lipoprotein A	135 (24-806)	82 (24-679)	*0.345*
% Body Fat	25.4 (19.4-36.5)	25.4 (21.0-37.2)	*0.028*
Waist:Hip Ratio	0.87 (0.83-1.03)	0.91 (0.86-0.97)	*0.398*
AGHDA (Max 25)	3 (0-23)	6 (2-23)	*0.194*
ACROQOL (Max 110)	84.1 (29.5-109.1)	78.4 (15.9-98.9)	*0.310*
EUROQOL	75 (50-95)	76 (50-95)	*0.465*

Figure 5.4 Change in Fasting Plasma Glucose in Response to Increased Pegvisomant Dosing

This graph represents the changes in fasting glucose in relation to a change in GH activity caused by the increase in pegvisomant dose. Each line corresponds to an individual patient's data; the baseline level was taken on the maintenance dose pegvisomant and the final level was taken on the increased pegvisomant dose used for the trial period. No statistically significant difference was seen with any parameter in this cohort; this is likely to reflect a combination of the difficulty in achieving the target sub-normal IGF-I levels and the modest numbers of patients available to recruit into such studies, even in tertiary centres.

One outlier is noted for the fasting glucose measurements; levels increased from 5.6mmol/l to 7.9mmol/l in this patient. Repeat analysis of the results excluding the outlier confirmed a lack of a statistically significance (p=0.091).

Figure 5.4 Fasting Plasma Glucose in Response to Increased dose of Pegvisomant

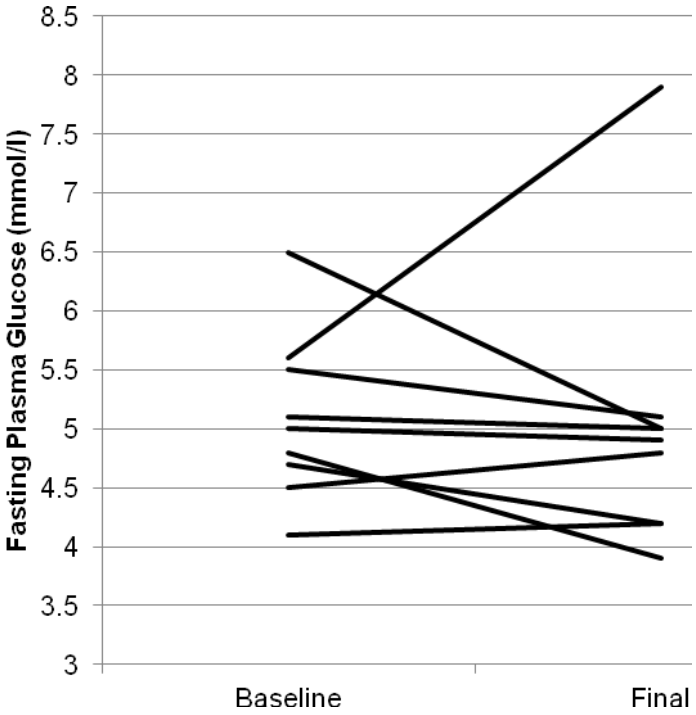


Figure 5.5 Change in Fasting Insulin levels in Response to Increased dose of Pegvisomant

This graph represents the changes in fasting insulin in relation to a change in GH activity caused by the increase in pegvisomant dose. Each line corresponds to an individual patient's data; the baseline level was taken on the maintenance dose pegvisomant and the final level was taken on the increased pegvisomant dose used for the trial period. No statistically significant difference was seen with any parameter in this cohort; this is likely to reflect a combination of the difficulty in achieving the target sub-normal IGF-I levels and the modest numbers of patients available to recruit into such studies, even in tertiary centres.

Figure 5.5

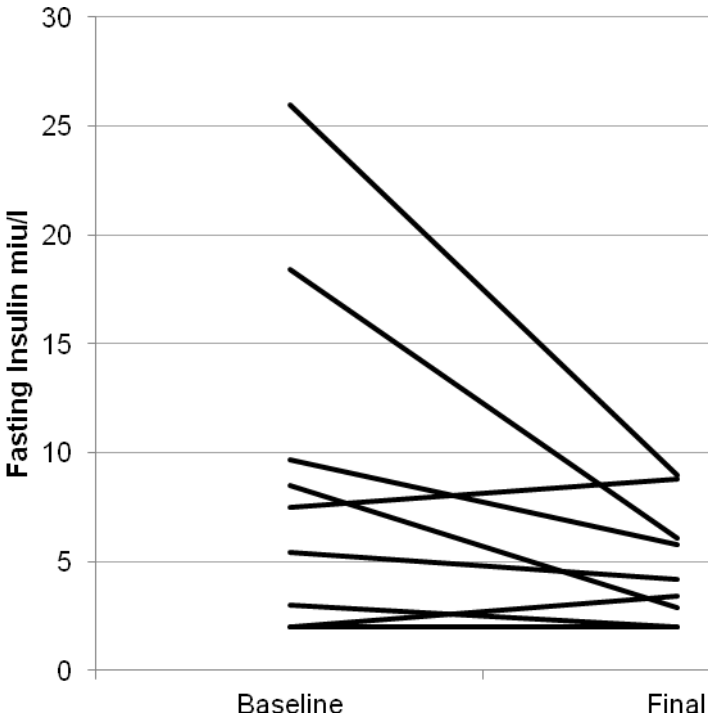


Figure 5.6 HOMA2-IR insulin resistance

This graph represents the changes in HOMA2 insulin resistance in relation to a change in GH activity caused by the increase in pegvisomant dose. Each line corresponds to an individual patient's data; the baseline level was taken on the maintenance dose pegvisomant and the final level was taken on the increased pegvisomant dose used for the trial period. No statistically significant difference was seen with any parameter in this cohort; this is likely to reflect a combination of the difficulty in achieving the target sub-normal IGF-I levels and the modest numbers of patients available to recruit into such studies, even in tertiary centres.

Figure 5.6

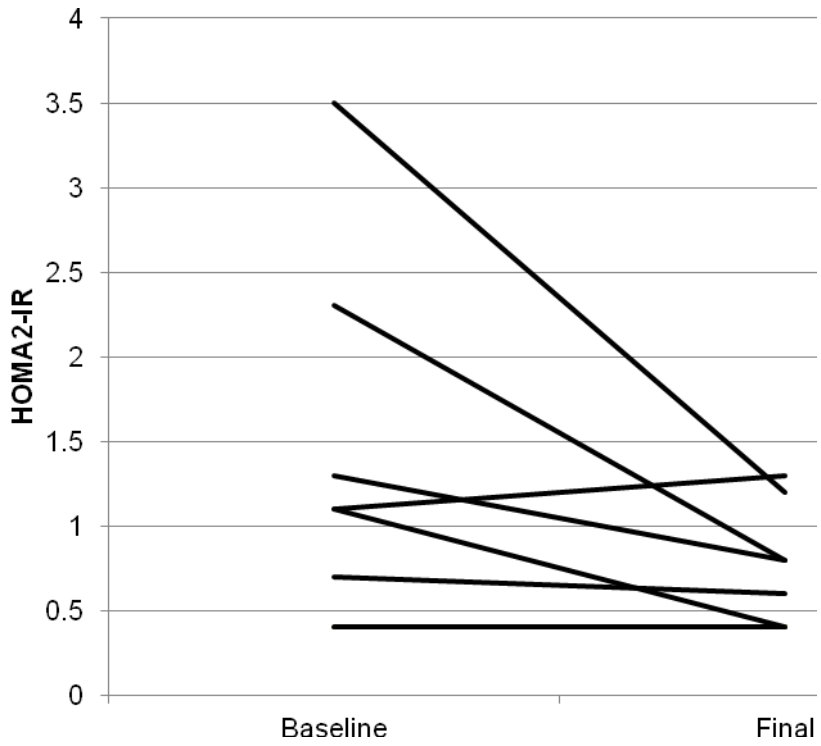


Figure 5.7 Change In Body Composition In Response To Increased Dose Pegvisomant

The following graph reflects the changes in body composition in response to the increased pegvisomant dose. %body fat was measured for each patient at baseline on their usual maintenance pegvisomant dose and again after 12 weeks on the increased trial dose; each line on the graph corresponds to an individual patient's data.

A statistically significant difference was detected for %body fat

Figure 5.7 Change in %Body Fat in Response to Increased Dose Pegvisomant

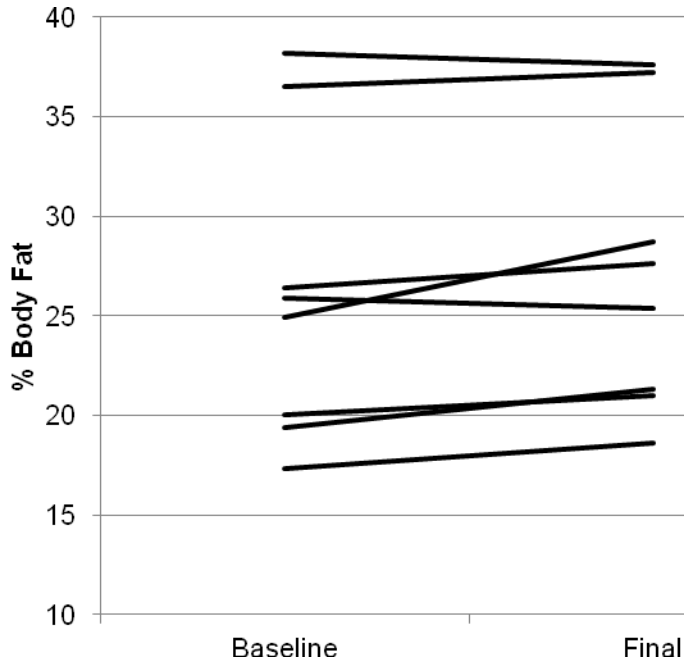


Figure 5.8 Change in Waist Hip Ratio in Response to Increased Dose Pegvisomant

The following graph reflects the changes waist:hip ratio in response to the increased pegvisomant dose; this was measured for each patient at baseline on their usual maintenance pegvisomant dose and again after 12 weeks on the increased trial dose; each line on the graph corresponds to an individual patient's data.

No statistically significant difference was detected.

Figure 5.8

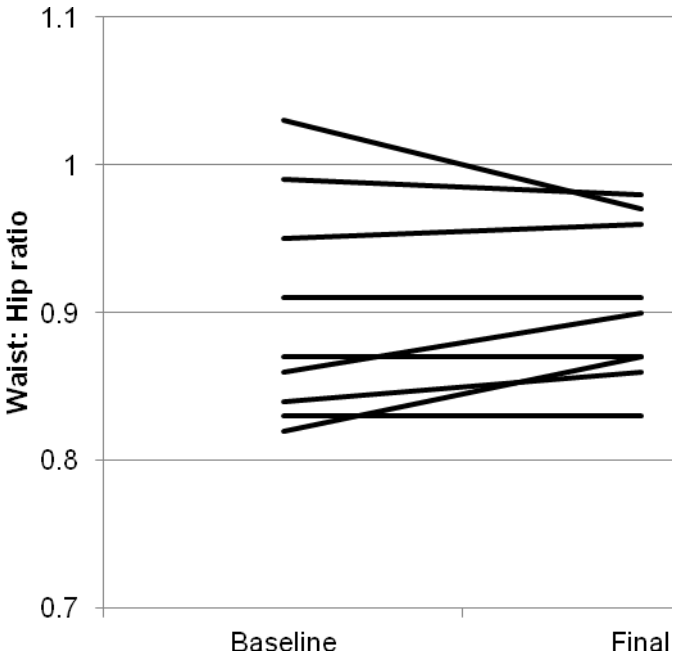


Figure 5.9 Change in Lipoprotein A levels in Response To Increased Dose Pegvisomant

The following graph reflects the changes in the cardiovascular risk marker lipoprotein A in response to the increased pegvisomant dose. These were measured for each patient at baseline on maintenance dose pegvisomant and again after 12 weeks on the increased trial dose; each line on the graph corresponds to an individual patient's data. No statistically significant difference was detected for lipoprotein a.

Figure 5.9 Change in Lipoprotein A levels in Response To Increased Dose Pegvisomant

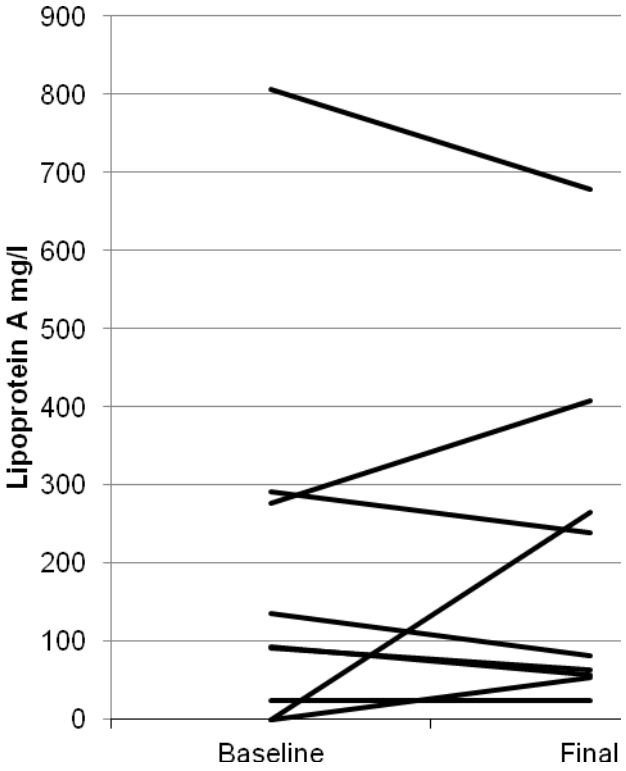


Figure 5.10 Change in Fibrinogen levels in Response To Increased Dose Pegvisomant

The following graph reflects the changes in the cardiovascular risk marker fibrinogen in response to the increased pegvisomant dose. These were measured for each patient at baseline on maintenance dose pegvisomant and again after 12 weeks on the increased trial dose; each line on the graph corresponds to an individual patient's data. A statistically significant difference was detected for fibrinogen.

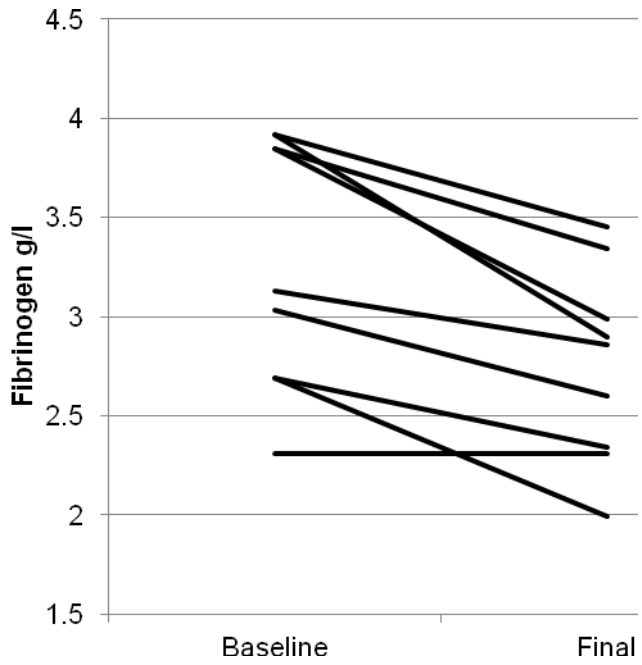


Figure 5.11 Changes in Adult Growth Hormone Deficiency Assessment (AGHDA) In Response To Increased Dose Pegvisomant

The following graph reflects the change in the AGHDA quality of life questionnaire score in response to the increased pegvisomant dose and consequent reduction in GH activity. These were measured for each patient at baseline on maintenance dose pegvisomant and again after 12 weeks on the increased trial dose; each line on the graph corresponds to an individual patient's data.

No statistically significant difference was detected between baseline and on the trial dose of pegvisomant.

Figure 5.11 Changes in Adult Growth Hormone Deficiency Assessment (AGHDA) In Response To Increased Dose Pegvisomant

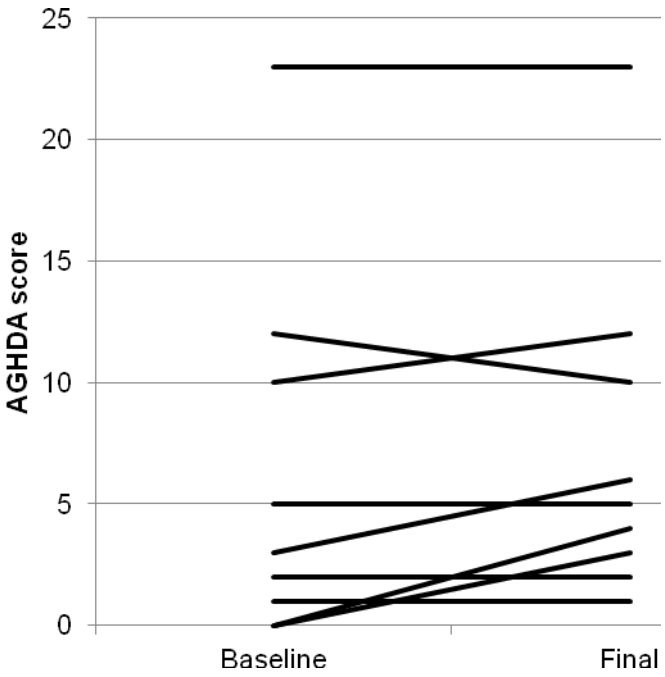


Figure 5.12 Changes in AcroQoL In Response To Increased Dose Pegvisomant

The following graph reflects the change in the AcroQoL quality of life questionnaire score in response to the increased pegvisomant dose and consequent reduction in GH activity. These were measured for each patient at baseline on maintenance dose pegvisomant and again after 12 weeks on the increased trial dose; each line on the graph corresponds to an individual patient's data.

No statistically significant difference was detected between baseline and on the trial dose of pegvisomant.

Figure 5.12

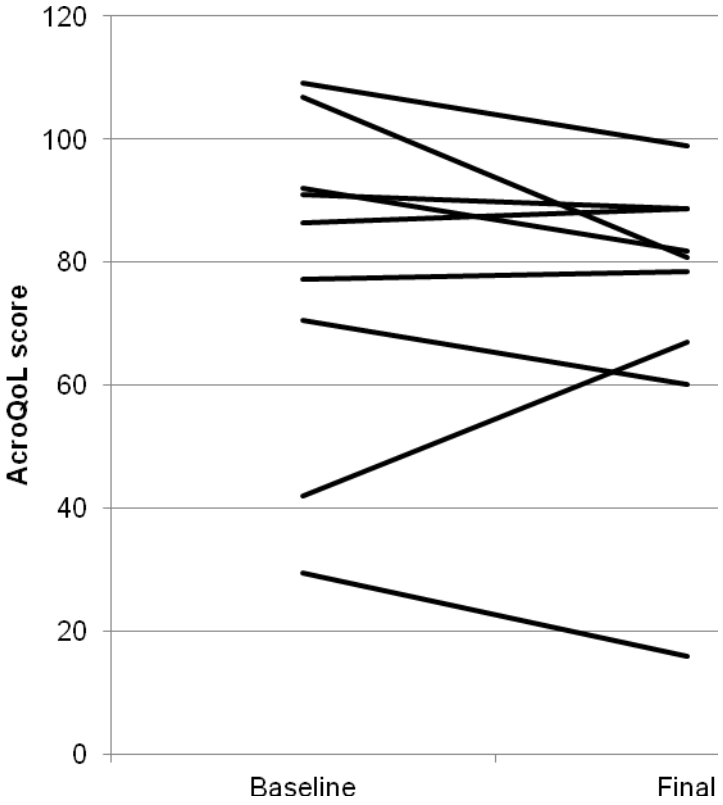


Figure 5.13 Change in EuroQoL Visual Analogue score In Response To Increased Dose Pegvisomant

The following graph reflects the change in the EuroQoL quality of life questionnaire score in response to the increased pegvisomant dose and consequent reduction in GH activity. These were measured for each patient at baseline on maintenance dose pegvisomant and again after 12 weeks on the increased trial dose; each line on the graph corresponds to an individual patient's data.

No statistically significant difference was detected between baseline and on the trial dose of pegvisomant.

Figure 5.13

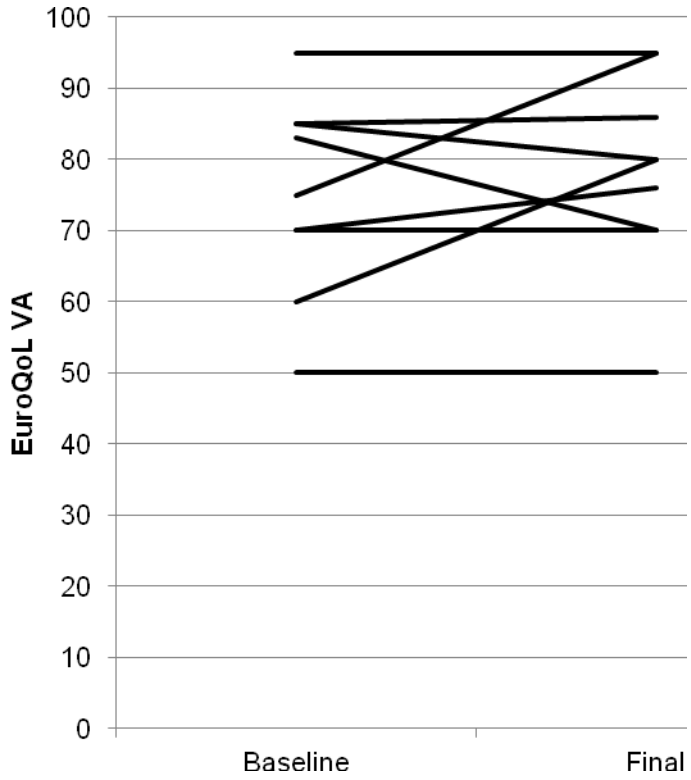
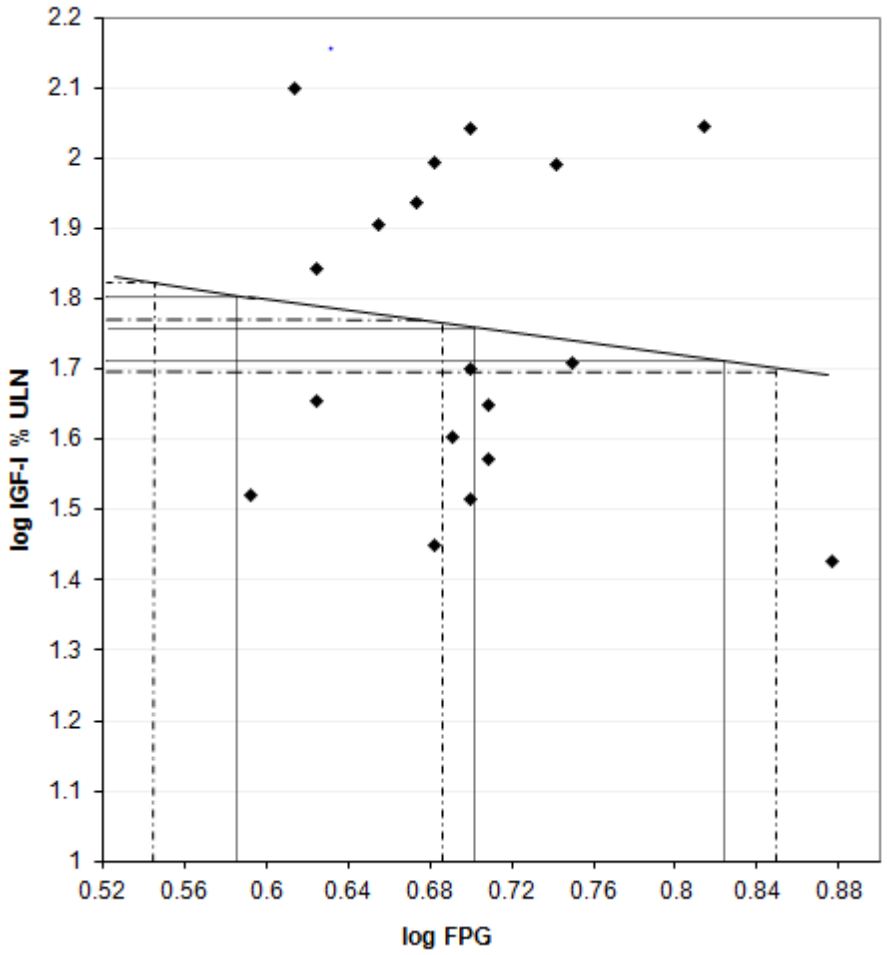


Figure 5.14 Plot of the Results for Fasting plasma glucose against IGF-I

This graph shows the plot of the log [fasting plasma glucose] results taken at the start of the trial on maintenance pegvisomant dose and at the end of the trial on an increased dose against log [IGF-I %ULN] . Log transformation was necessary to form a straight line relationship and the regression line is shown. The dotted lines(····) demonstrate the mean +/- 1.96x SD for baseline FPG results. The dashed lines(- - -) demonstrate the mean +/- 1.96x SD for the final FPG results.

The intention of the study was to render patients mildly GH deficient in order to investigate the effect on metabolic markers such as FPG to try to narrow the range of IGF-I as a guide for treatment, by calculating the difference between mean +/- 1.96 x standard deviation on the samples at baseline and at trial end. Unexpectedly even in spite of maximal doses of pegvisomant the majority of the cohort failed to lower their IGF-I levels sufficiently, thereby resulting in a significant overlap in metabolic marker results such as FPG. This overlap of results prevents the calculation of the “safe range” of IGF-I.

Figure 5.14 Plot of the Results for Fasting plasma glucose against IGF-I



5.5 Discussion

Pegvisomant is well established as a medical therapy for patients with acromegaly whose disease is refractory to conventional therapies, but its clinical use is made difficult by the lack of a robust method for monitoring treatment. Although serum IGF-I serves as a marker of GH activity, variability in the assay and known discordance between GH and IGF-I levels limit its reliability. The original purpose of this study was to investigate, using biological parameters relevant to the GH/IGF-I system, whether there is an 'optimum part of the age-adjusted IGF-I reference range within which clinicians can be confident that patients are not either over- or under-treated.

The main conclusion of this study was the unexpected finding that even with maximal increases in pegvisomant dose it was difficult to reduce serum IGF-I to levels below the reference range. This observation hampered the planned statistical analyses but did provide an interesting insight into the pharmacological properties of pegvisomant.

Summary of Results

Response to Pegvisomant Dose Titration

The cohort of nine patients underwent a scheduled dose titration whereby doses were increased by 2 to 4.6 fold. Only three patients achieved a target IGF-I of below the age-related reference range, all of whom were female and two of whom were postmenopausal, suggesting that this observation is unlikely to be an oestrogen effect. Previous data suggests that increased body mass is associated with increased pegvisomant clearance; to test this hypothesis a regression analysis of

ΔIGF-I was performed to investigate the effects of body weight, gender and pegvisomant dose. None of these factors were found to have a significant effect on ΔIGF-I.

It should be noted that although six patients failed to achieve the target IGF-I of below the normal reference range, all patients achieved a reduction in serum IGF-I of between 26.7 and 66.4%. Those who failed to reach the target range had higher starting values and so had further to fall; it is possible that further increases in pegvisomant doses may have been able to achieve the desired subnormal serum IGF-I, but a lack of safety data regarding the use of higher daily doses constrained the protocol design. It should also be noted that although all patients prior to enrolment had serum IGF-I levels within the normal range, values at commencement of the dose titration protocol were actually above the normal reference range for three patients. Although they subsequently achieved between 44 - 63% reductions in IGF-I, they required a far larger drop in order to achieve target IGF-I.

Effect of Pegvisomant on Physiological Markers of GH activity

Due to the lack of overt 'pharmacological' GH deficiency (arbitrarily defined as a subnormal serum IGF-I) in the majority of the cohort, the original statistical analyses of these results were not possible. All measures assessed are known to be markers of GH activity with distinct patterns in relation to both GH excess and deficiency. Non-parametric testing confirmed that there was not a statistically significant difference between baseline and final results for the majority of the physiological markers – presumably due, at least in part, to this difficulty in achieving a sufficiently low serum IGF-I.

On review of individual results, some marked improvements in metabolic markers occurred in response to the reduction in IGF-I. Patient no 6's fasting insulin level decreased from 26miu/l to 9miu/l with a reduction in fasting glucose from 6.5 to 5mmol/l as a result of reducing serum IGF-I from the upper limit of normal to the level of the median for his age related reference range. This emphasises the point that a clearer target for treatment is needed in order to minimise risk of excess GH; prior to the study he was considered to be on a satisfactory dose of pegvisomant but these results suggest it is entirely plausible that values of serum IGF-I that lie within a population reference may represent marginal GH excess for an individual patient.

Such exuberant responses were not universal throughout the cohort for each measure however, thus accounting for the lack of statistical significance. The quality of life questionnaires in particular demonstrated a variety of responses which emphasise the potential for bias. It is not possible to remove the influence of an individual's personality when using these quality of life scores. For example one patient scored 23/25 on AGHDA questionnaires both at baseline and at the end of the study; no change was evident despite the IGF-I level decreasing from above the median to a level of GHD (IGF-I 64ng/ml).

Critique of Work

The unexpected difficulty in achieving the target IGF-I is a major drawback of this study; as such the proposed statistical analyses were not possible. The protocol was written using all available pharmacological data from the original pegvisomant trials; however the previous studies had not intended to cause GH deficiency and therefore the relevant information regarding high dose pegvisomant was not available. The study was also limited by the lack of safety and pharmacokinetic data

above 50mg; without such data it was not possible to obtain the necessary approval to use higher doses for the purpose of a study.

In order to fully answer the study question of identifying the optimum serum IGF-I range with pegvisomant use, a number of requirements exist. This study design largely focused on improving the accuracy of the lower end of the normal IGF-I reference range by inducing pharmacological GH deficiency. Ideally the baseline investigations would occur at a time of GH excess followed by a period of mild GH deficiency in order to narrow the IGF-I range from the top and the bottom. This however is not possible in view of the ethical implications of withholding necessary treatment for GH excess with the potential for causing significant morbidity. This study was also restricted by the limitations on pegvisomant dose; it is possible that with further increases in dose all patients may have achieved target IGF-I although the lack of safety and pharmacokinetic data in humans above 50mg per day precludes this. A larger cohort would also have been preferable although cohort size would always be limited by the comparatively small number of patients on pegvisomant.

Other limitations of this work include the reproducibility of some measures. Waist hip ratio in particular is subject to error even when performed by the same investigator. Quality of life questionnaires are subject to influence by an individual's personality and judgement of their own symptoms; a more stoic individual tends to score lower marks than a person with depression and yet there is no method for correcting for this when analysing results. The duration of this study also influenced results, particularly as the dose titration phase took considerably longer than expected, there was up to 22 weeks between baseline and final assessments which had potential implications for the results. One outlier is evident in the fasting plasma glucose levels; results increased from 5.6 to 7.9mmol/l despite the reduction in GH

activity; this suggests that lifestyle factors perhaps influenced by the duration of the study affected this individual's insulin resistance.

Consideration of these data in the context of the Published Literature

No previous studies have sought to answer the question of identifying the optimal serum IGF-I for acromegalic patients treated with pegvisomant. Furthermore no previous studies have aimed to induce GH deficiency with pegvisomant or have used such high doses for an extended period of time.

There is a report of an accidental pegvisomant overdose; 80mg daily was taken for seven days instead of 80mg per week. The only symptom reported was slight increase in fatigue and the serum IGF-I decreased from 940ng/m to 153ng/ml (this measurement was taken after 4 weeks on the increased dose) (Pegvisomant Investigator's Brochure).

Future work

The experience gained from this study has given a further insight into the properties of pegvisomant that encourage further investigation. In order to further investigate the optimum IGF-I range ideally further studies would be undertaken using higher pegvisomant doses and in particular to perform more detailed pharmacokinetic studies of high dose pegvisomant. Previous data assumes a linear dose response relationship but this is based primarily on doses of 10-20mg per day.

It would also be preferable to perform the study prospectively with the baseline data taken prior to the commencement of pegvisomant, when IGF-I levels for the whole

cohort were high. This would allow a more robust analysis of the data with a clearer identification of the optimum IGF-I range rather than focusing primarily on the lower end of the normal reference range. The feasibility of such a study would however be limited by ethical considerations and the necessary time to recruit patients at a time of GH excess prior to commencement of pegvisomant.

The variability in achieving target IGF-I highlights the need for further studies into variability in pegvisomant responsiveness; factors such as heterogeneity in the GH receptor and variation in serum pegvisomant levels, in particular pegvisomant clearance in relation to body mass would be of interest.

Summary and Conclusion

Although the difficulty in achieving the target serum IGF-I in the cohort and to therefore perform the necessary statistical analyses was disappointing, this study has instead revealed the unpredicted difficulty in achieving sub-normal IGF-I levels with pegvisomant. Such information is of interest to the clinician; it does provide some reassurance that it is surprisingly difficult to over-treat a patient with pegvisomant. It has also highlighted the variability that is seen with pegvisomant levels and raises questions over what is underlying this variability in response.

Chapter 6

Final Discussion

Clinical endocrine practice frequently involves ‘fine tuning’ therapy according to an individual patient’s needs. Hormonal assays can be useful in this regard. In primary and secondary adrenal failure, for example, measurements of serum cortisol (a reasonably robust assay in most hospital laboratories) may aid clinical decisions regarding glucocorticoid dosing. Similarly, measurements of serum testosterone are a useful adjunct to reported symptoms when deciding on a male patient’s replacement regimen for primary or secondary hypogonadism. In disorders of the GH/IGF-I axis, clinical decision making can sometimes be difficult. Clear epidemiological data exist that provide ‘target’ ranges for GH and IGF-I in the treatment of acromegaly, but what should the physician advise if the values in an individual are discordant?

This thesis has attempted to explore various aspects of variability in GH signal transduction into IGF-I that remain poorly understood. An improved understanding of the underlying mechanisms may help with clinical decision making and has the potential for tailoring clinical treatment to the individual.

The obvious candidate is variability in the GH receptor; the exon 3 deletion within the GHR has been extensively investigated in paediatric and adult populations but without conclusive evidence for or against a significant role in GH responsiveness. The majority of previously published studies combined d3-GHR homo and heterozygotes for analysis; it is possible that this was responsible for masking the true effect of this polymorphism. Furthermore, the majority of previously published studies did not perform the repeat PCR analysis to exclude the possible misclassification of d3-GHR heterozygotes as homozygotes. The studies of GHD and acromegalic patients within this thesis aimed to definitely answer the question of whether the d3-GHR polymorphism significantly influences GH signal transduction into IGF-I.

Detailed analyses of large cohorts of 194 GHD patients and 79 acromegalic patients were performed, separately analysing d3 homo- and heterozygotes and ensuring the extra PCR was performed. Despite these extra measures to improve the sensitivity of these studies to detect a difference in GH responsiveness, the possession of one or more d3-GHR allele was not found to significantly augment GH response. In the GHD population, only a marginal difference was detected in ΔIGF-I at 12 months, with no difference detected at 6 months or in rhGH dose used. The potential benefit of identifying GH responsiveness factors would be to provide a more tailored approach to treatment. These data suggest that it is not something that is of clinical relevance.

In the acromegalic population, neither d3-GHR homo nor heterozygosity was found to have a significant effect on the relationship between GH and IGF-I. Again this would suggest that GHR variability is not something that needs to be factored into clinical assessment or decision making.

Cumulatively, these results in conjunction with previously published data suggest that the d3-GHR polymorphism does not significantly affect GHR signal transduction and there is little therapeutic benefit in determining an individual's d3-GHR status. Other factors relevant in determining GH signal transduction into IGF-I must exist and further work is needed to identify these. The need for sensitive and reproducible markers of GH activity, particularly for the adult population, to complement measurements of serum IGF-I is evident.

The insensitivity of IGF-I as an accurate marker of GH activity is also of particular importance with pegvisomant use; this is the sole measure used to guide pegvisomant dose. The work within this thesis aimed to identify an optimum range of IGF-I within which metabolic parameters returned to normal and provide useful information for physicians regarding the avoidance of pharmacological GHD during

pegvisomant treatment. Unpredictably, it proved to be difficult to induce pharmacological GHD even with maximal doses of pegvisomant. Pegvisomant was presumed to have a linear dose response relationship based on original pharmacokinetic data using the standard lower therapeutic doses of between 10-40mg per day. No previous study has ever aimed to induce pharmacological GHD or used doses of pegvisomant described here. This work has, for the first time, demonstrated the effects of high dose pegvisomant and has shown that there appears to be some variability in response to this drug. What is causing this variability in pegvisomant responsiveness is not clear; variability within the GH receptor is likely to play a part, along with variation in clearance rates related to body mass but other potential factors including the role of the growth hormone binding protein are of potential relevance. Further studies to determine the underlying reasons for variability in pegvisomant responsiveness are warranted.

One of the major problems identified by this thesis is the use of IGF-I as the predominant marker of GH activity in the adult population; the wide normal reference range, variability in the assay and the impact of factors such as insulin resistance and oestrogen on IGF-I generation limit it's sensitivity and usefulness. To allow full, detailed investigation of GH signal transduction of GH into IGF-I, the reliability of IGF-I assay needs to improve. This is however a chicken and egg situation; without identifying the reasons for the variability in GH signal transduction into IGF-I, the sensitivity of IGF-I as a research tool cannot improve. But until either the reliability of serum IGF-I improve or an alternate marker is found the variability in GH signal transduction into IGF-I cannot be fully and accurately investigated in the adult population. Despite massive improvements in assay technology and

availability, the practice of clinical endocrinology still requires a substantial amount of clinical judgement.

Future Work

The underlying reasons for variability in GH responsiveness remain unresolved and more work is needed to identify these in both adult and paediatric populations. The exon 3 deletion has been investigated extensively without a meaningful conclusion; this suggests that other, more important factors exist. It is likely that other polymorphisms within the GH receptor although, to the author's knowledge, to date none have been discovered. Future work should initially focus on the -202 A polymorphism of IGFBP3; there is evidence within the paediatric population that this enhances IGFBP3 levels and growth velocity (Costalonga, Antonini et al. 2009). A more recent study however, has showed that there was no effect on final height achieved by 178 subjects with severe GH deficiency in childhood compared with a control group(Miletta, Scheidegger et al. 2012). The effect of this polymorphism within adult populations has not yet been investigated; this will form the focus of future studies into GH responsiveness. Although, as noted previously, further work on the sensitivity of the IGF-I assay is also needed to optimise our abilities to detect subtle changes in GH responsiveness.

Publications, Abstracts and Prizes

Publications

VJ Moyes, DM Walker, S Owusu-Antwi, KT Maher, L Metherell, SA Akker, JP Monson, AJL Clark, WM Drake (2010) d3-GHR Genotype Does Not Explain Heterogeneity In GH Responsiveness In Hypopituitary Adults
Clin Endocrinol (Oxf). 2010 Jun;72(6):807-13.

Published Abstracts

Oral Presentation
VJ Moyes, DM Walker, S Owusu-Antwi, KT Maher, L Metherell, SA Akker, JP Monson, AJL Clark, WM Drake. d3-GHR Genotype Does Not Explain Heterogeneity In GH Responsiveness In Hypopituitary Adults
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Poster Presentation
VJ Moyes, DM Walker, S Owusu-Antwi, KT Maher, L Metherell, SA Akker, JP Monson, AJL Clark, WM Drake. d3-GHR Genotype Does Not Explain Heterogeneity In GH Responsiveness In Hypopituitary Adults
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Prizes

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d3-GHR Genotype Does Not Explain Heterogeneity In GH Responsiveness In Hypopituitary Adults.
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Appendix

Queen Mary University of London

Barts and The London NHS NHS Trust

Patient Information Sheet

Effect of Growth Hormone Genotype on Growth Hormone responsiveness in patients with Growth Hormone Deficiency

Lay Title: Why do some patients with growth hormone deficiency respond better than others to growth hormone replacement?

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Background:

As you may know, growth hormone is important for adults as well as children as it has important effects on the body including cholesterol levels, sense of well-being and fat distribution. When patients are lacking in growth hormone we aim to replace it with daily injections of growth hormone. When we replace growth hormone it is important that we closely monitor treatment to ensure we achieve our target, but also to ensure we do not give too high a dose as this may cause different types of problems. We do this by checking the level of a hormone called IGF1; this is produced by the liver in response to growth hormone. When patients are deficient in

growth hormone their IGF1 levels are usually low and when patients have too much growth hormone their IGF1 levels are usually high.

Our department has a lot of experience in treating patients with growth hormone and we have noticed that the doses required to achieve the target normal IGF1 level varies considerably between patients and we do not know why. We would like to investigate the possible reasons for this, so that we can learn how to select the best dose of growth hormone for each patient. We believe that the likely cause for this variability is the growth hormone receptor. A hormone acting through a receptor is rather like a key fitting into a lock: the key has to be a good fit for it to work. A recent study in children has suggested that different people may have slightly different growth hormone receptors on their cells. Growth hormone fits into these different receptors slightly differently and this appears to explain why some short children who are treated with growth hormone grow more than others. It is possible that the same differences in the growth hormone receptor are the explanation for the differences in adult patients' response to growth hormone.

<u>What it involves:</u>

When you have your next routine blood test on Francis Fraser ward, we would like to take an extra 10mls (1 tablespoon) of blood so that we can test it and see which type of growth hormone receptor you have. This is done by looking at the part of your DNA that is responsible for the growth hormone receptor. DNA is the genetic material within the cell and is divided into functional units called genes which carry the instructions for making up the body. Each gene relates to a specific part of the body and we are only interested in the genes responsible for the growth hormone

receptor, we will not be looking at the genes responsible for any other disease. This blood test is your only direct involvement in this study.

We already have very clear records in your notes of the other information we need. In particular we will be looking at the dose of growth hormone you require and how your IGF1 level has changed on treatment. We will also be looking at the other measurements that we regularly record as part of the monitoring of your treatment; cholesterol levels, waist:hip ratio, and how your quality of life score has improved on treatment. We will also be looking at whether previous treatment such as pituitary radiotherapy and the presence or absence of oestrogen (i.e. whether you are male or female) has an effect on your results. All of this data is already in your notes and so will not be repeated for the study.

Do I have to take part?

Participation in the study is entirely voluntary; it is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. Any stored blood or tissue samples that can still be identified as yours will be destroyed on completion of the study.

What will happen if you take part?

We will need to arrange to meet in order for the consent form to be signed and any additional questions may be asked. We will then make arrangements for your blood test to be taken when you next attend our department. No further direct involvement is needed from you, all other results have already been documented in your notes

since you commenced on GH. Your clinical care will continue within the Department of Endocrinology at St. Bartholomew's Hospital.

What are the other possible disadvantages and risks of taking part?

Other than the discomfort of having a blood test (which will be taken at the time of a routine blood test anyway) we do not perceive there to be any other risks or disadvantages to the participants. Genetic analysis for the DNA responsible for the GH receptor will not affect your ability to obtain life or medical insurance, but if you do experience any problems as a result of this study, the insurance company may contact the research team for verification.

Possible benefits of participation

We cannot promise the study will help you but the information we get might help improve the treatment of people with growth hormone deficiency in the future.

Results

We plan to publish the results of this study in a medical journal and as part of an MD thesis in due course. No identifiable patient details will be included in any publication (i.e. no names, date of birth etc) unless prior consent from each patient has been obtained.

This study is being funded by Ipsen, a pharmaceutical company who make one particular brand of GH. They are interested in the results of this study as it will improve the general understanding of GH replacement. As a result of this sponsorship they will have access to our study data and results, but they will not have access to any of your personal details. The study has been designed and will be run entirely by doctors working in the Department of Endocrinology at Barts and the London Trust.

What happens to the blood samples?

We will extract your DNA and analyse the part responsible for the GH receptor. If the results of the study do not explain why there is such variability in growth hormone response we may wish to test other genes that may provide the answer during the course of this study. No analysis of your DNA will take place that is not related to how growth hormone signals into IGF-I. Once the study has completed, we will destroy all samples.

What happens to all data?

The required clinical data (blood results etc) will be obtained from your hospital notes and all data will be stored in a file within a Barts and the London trust computer that will only be accessible by members of the research team.

If you join the study, some parts of your medical records and the data collected for the study may be looked at by representatives of regulatory authorities and by authorised people from Barts and the London Trust, other NHS bodies to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

All information which is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research the people conducting the study will abide by the Data Protection Act 1998, and the rights you have under this Act.

With your consent we will inform your GP of your participation in this study but specific details regarding your results will not be disclosed without your consent.

What happens if there is a problem?

We would not expect you to suffer any harm or injury because of your participation in it. If you are harmed by taking part in this study, there is no special compensation arrangement. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay your legal costs. Regardless of this, if you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Please contact Patient Advisory Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint. Please telephone 020 7377 6335, minicom 020 7943 1350, or email pals@bartsandthelondon.nhs.uk, you can also visit PALS by asking at any hospital reception.

What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, you will be told why and your continuing care will be arranged.

What happens when the research study stops?

You will continue to receive your usual care at the endocrinology department at St Bartholomew's Hospital throughout the course of this study and after it concludes.

What if there is a problem and Contact Details for further information

Further details can be obtained from:

Dr V Moyes or Dr W Drake

Department of Endocrinology

St Bartholomew's Hospital

West Smithfield

London EC1A 7BE

0207 601 8346 or via Barts and the London switchboard 0207 377 7000

email: veronica.moyes@bartsandthelondon.nhs.uk

Who is organising and funding the research and where was it reviewed?

This project has been organised by Dr WM Drake with Ipsen Ltd and has been peer reviewed. This study was given a favourable ethical opinion for conduct in the NHS by the East London and City REC

Queen Mary University of London

Barts and The London NHS Trust

CONSENT FORM (Version 3 Dated 18/10/2007)

Effect of Growth Hormone Genotype on Growth Hormone responsiveness in patients with Growth Hormone Deficiency

Investigator: Dr WM Drake, Dr VJ Moyes

Centre Number: Patient Identification Number for this trial:

Please initial box to indicate agreement

1.	I confirm that I have read and understand the information sheet: Effect of Growth Hormone Genotype on Growth Hormone responsiveness in patients with Growth Hormone Deficiency, dated 18/10/7 (version 7) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the Barts and the London/ Queen Mary University of London, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4.	I agree to my GP being informed of my participation in the study.	
5.	I agree to take part in the above study.	
6.	I understand that my blood sample will be destroyed on completion of the study.	

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from Investigator)	Date	Signature
_____	_____	_____
Investigator	Date	Signature

1 copy for Patient, 1 for Investigator and original to be kept in medical notes

Queen Mary University of London

Barts and The London NHS Trust

Patient Information Sheet

Effect of Growth Hormone Genotype on Growth Hormone responsiveness in patients with Acromegaly

Lay Title: Why do some patients with growth hormone excess (acromegaly) need more therapy than others to achieve their target blood tests?

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of our study?

In adults, too much growth hormone causes a condition called acromegaly, which leads to unpleasant symptoms including sweating, joint pain and fatigue and overgrowth of soft tissues. When doctors are diagnosing and assessing acromegaly, they usually measure two blood tests: the level of growth hormone and also the level of insulin-like growth factor I (IGF-I). Growth hormone triggers the production of IGF-I and so in general the more growth hormone there is in the bloodstream the more IGF-I will be made and the worse a patient's symptoms will be. Previous studies have shown that for most patients there is a direct relationship between level of growth hormone and level of IGF1, however in about 1/3 of patients there is no clear correlation. This can work both ways, for some patients the IGF-I level is lower than expected for the amount of growth hormone whilst for other patients rather low growth hormone levels are associated with a high IGF-I. We

would like to understand why this happens as it will help us to be more accurate when we treat patients with acromegaly.

Growth hormone, like all hormones, is a chemical messenger and it has its effect by fitting into the growth hormone receptor which is present on virtually all cells of the body. A hormone acting through a receptor is rather like a key fitting into a lock: the key has to be a good fit for it to work. A recent study in children has suggested that different people may have slightly different growth hormone receptors on their cells. Growth hormone fits into these different receptors slightly differently and this appears to explain why some short children who are treated with growth hormone, grow more than others. It is possible that the same differences in the growth hormone receptor form part of the explanation as to the discrepancy between growth hormone and IGF1 levels in some patients with acromegaly.

What it involves:

When you have your next routine blood test on Francis Fraser ward, we would like to take an extra 10mls (1 tablespoon) of blood so that we can test it and see which type of growth hormone receptor you have. This is done by looking at the part of your DNA that is responsible for the growth hormone receptor. DNA is the genetic material within the cell and is divided into functional units called genes which carry the instructions for making up the body. Each gene relates to a specific part of the body and we are only interested in the genes responsible for the growth hormone receptor, we will not be looking at the genes responsible for any other disease. This blood test is your only direct involvement in this study. We already have very clear records in your notes of the other information we need. In particular we will be looking at your growth hormone levels and IGF1 results, all of which is already documented in your notes. We will also collect data regarding your cholesterol and insulin levels, which is also documented within your notes.

Do you have to take part?

Participation in the study is entirely voluntary; it is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

You can withdraw from treatment but keep in contact with us to let us know your progress. Information collected may still be used. Any stored blood or tissue samples that can still be identified as yours will be destroyed on completion of the study.

What will happen if you take part?

We will need to arrange to meet in order for the consent form to be signed and any additional questions may be asked. We will then co-ordinate the additional blood tests to be withdrawn at the time of your next routine blood test on Francis Fraser ward. We also obtain results of fasting glucose and insulin (can be from previous blood tests if results available) and markers of body composition.

Your clinical care will continue as usual within the Department of Endocrinology at St. Bartholomew's Hospital.

Possible benefits of participation

We cannot promise the study will help you but the information we get might help improve the treatment of people with acromegaly in the future.

What are the other possible disadvantages and risks of taking part?

Other than the discomfort of having a blood test (which will be taken at the time of a routine blood test anyway) we do not perceive there to be any other risks or disadvantages to the participants. Genetic analysis for the DNA responsible for the GH receptor will not affect your ability to obtain life or medical insurance, but if you do experience any problems as a result of this study the insurance company may contact our research team for verification.

Results

We plan to publish the results of this study in a medical journal and as part of an MD thesis in due course. No identifiable patient details will be included in any publication (ie names, date of birth etc) unless prior consent from each patient has been obtained.

This study is being funded by Ipsen, a pharmaceutical company who make one of the medical treatments available for the treatment of acromegaly. As a result of this sponsorship they will have access to our study data and results, but they will not have access to any of your personal details. The study has been designed and will be run entirely by doctors working in the Department of Endocrinology at Barts and the London Trust.

What happens when the research study stops?

You will continue to receive your usual care at the endocrinology department at St Bartholomew's Hospital throughout the course of this study and after it concludes.

What happens to the blood samples?

We will extract your DNA and analyse the part responsible for the GH receptor. If the results of this study do not explain why growth hormone and IGF-I levels are discrepant in some patients, we may wish to test other genes that may provide the

answer, during the course of this study. No analysis of your DNA will take place that is not related to how growth hormone signals into IGF-I. Once the study has completed, we will destroy all samples.

What happens to all data?

The required clinical data (blood results etc) will be obtained from your hospital notes and all data will be stored in a file within a Barts and the London trust computer that will only be accessible by members of the research team.

If you join the study, some parts of your medical records and the data collected for the study may be looked at by representatives of regulatory authorities and by authorised people from Barts and the London Trust, other NHS bodies to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

All information which is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research the people conducting the study will abide by the Data Protection Act 1998, and the rights you have under this Act.

With your consent, we will inform your GP of your participation in this study but specific details regarding your results will not be disclosed without your consent.

What happens if there is a problem?

We would not expect you to suffer any harm or injury because of your participation in it. If you are harmed by taking part in this study, there is no special compensation arrangement. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay your legal costs. Regardless of

this, if you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Please contact Patient Advisory Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint. Please telephone 020 7377 6335, minicom 020 7943 1350, or email pals@bartsandthelondon.nhs.uk, you can also visit PALS by asking at any hospital reception.

What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, you will be told why and your continuing care will be arranged.

What if there is a problem and Contact Details for further information

Further details can be obtained from:

Dr V Moyes or Dr W Drake

Department of Endocrinology,

St Bartholomew's Hospital

West Smithfield

London EC1A 7BE

0207 601 7706 or via Barts and the London switchboard on 0207 377 7000

email: veronica.moyes@bartsandthelondon.nhs.uk

Who is organising and funding the research and where was it reviewed?

This project has been organised by Dr WM Drake with Ipsen Ltd and has been peer reviewed. This study was given a favourable ethical opinion for conduct in the NHS by the East London and City REC

Queen Mary University of London

Barts and The London NHS Trust

CONSENT FORM (Version 3 Dated 18/10/2007)

Effect of Growth Hormone Genotype on Growth Hormone responsiveness in patients with Acromegaly

Investigator: Dr WM Drake, Dr VJ Moyes

Centre Number: Patient Identification Number for this trial:

Please initial box to indicate agreement

1.	I confirm that I have read and understand the information sheet: Effect of Growth Hormone Genotype on Growth Hormone responsiveness in patients with Acromegaly, dated 18/10/7 (version 7) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the Barts and the London/ Queen Mary University of London, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4.	I agree to my GP being informed of my participation in the study.	
5.	I agree to take part in the above study.	
6.	I understand that my blood sample will be destroyed on completion of the study.	

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from Investigator)	Date	Signature
_____	_____	_____
Investigator	Date	Signature

1 copy for Patient, 1 for Investigator and original to be kept in medical notes

Queen Mary University of London

Barts and The London NHS NHS Trust

Patient Information Sheet

A Study to Determine the Optimum Serum IGF-I Range in Patients with Acromegaly Treated with Pegvisomant.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of our study?

Pegvisomant is a relatively new drug and as a result of the way it works, the only way we can monitor treatment with pegvisomant is with the IGF1 level- a blood test that acts as a marker of growth hormone activity. Growth hormone levels cannot be measured as pegvisomant is a similar shape to growth hormone and so it is not possible to discriminate between the two with the current biochemical tests. We know from patients with acromegaly and also those with growth hormone deficiency, that IGF1 levels are not completely reliable in a proportion of patients. Monitoring of treatment with pegvisomant can therefore be difficult and there is a possibility that there are some patients on pegvisomant who are either under- or over-treated as a result of this. We would like to involve you in a study that will help us to understand what levels of IGF1 we should be aiming for to ensure that all patients are receiving just the right amount of pegvisomant. If we can work out the best levels to be aiming for this will not only help us but also will act as a guide for other doctors looking after patients on pegvisomant.

What it involves:

If you agree to participate in the study we will meet in person to discuss any queries you may have and you will need to sign a consent form.

In order for you to participate in this study, it is a requirement that you are already on a stable dose of pegvisomant. At the start of the study we will perform a number of assessments that demonstrate various aspects of your metabolism whilst on your usual dose of pegvisomant. These are:

- Blood tests: 2 single blood tests and a series of 5 blood samples performed over 3 hours to measure your response to glucose and/or insulin. The total amount of blood taken during these tests is approximately 20-30mls, equivalent to 2 tablespoons of blood.
- 24 hour urine collection to measure substances called cortisone/cortisol, the balance of which is partly regulated by GH and IGF1 levels
- Further measurements of IGF-I, two weeks after each change in dose of pegvisomant, until you reach the target level.
- Body composition assessments with measurements of your waist and hips and by performing a DEXA scan that helps to determine your body composition.
- Quality of life questionnaires

Your dose of pegvisomant will then be increased (on average by 10-15mg) in order to slightly over-treat you. We will measure your IGF1 level (5ml blood test) that acts as a marker of the activity of your acromegaly and we are aiming for just below the normal range. Once we have achieved a suitable IGF1 level, we will continue this dose for 12 weeks and at the end of this time we will repeat the above measurements. By determining how the above measurements change and pin-pointing when they change in relation to your IGF1 level, we will be able to determine the optimal range of IGF1 that we should be aiming for in the treatment with pegvisomant.

Do you have to take part?

Participation in the study is entirely voluntary; it is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. Information collected may still be used. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

Results

We plan to publish the results of this study in a medical journal and as part of an MD thesis in due course. No identifiable patient details will be included in any publication (i.e. names, date of birth etc) unless prior consent from each patient has been obtained.

Pfizer, the pharmaceutical company who make pegvisomant are providing funding for this study. As a result of this sponsorship they will have access to our study data and results, but they will not have access to any of your personal details. The study has been designed and will be run entirely by doctors working in the Department of Endocrinology at St. Bartholomew's Hospital.

Possible benefits of participation

We cannot promise the study will help you but the information we get might help improve the treatment of people with acromegaly in the future.

What are the other possible disadvantages and risks of taking part?

This study will involve injecting larger doses of pegvisomant than you normally take, and so this may require more than one injection per day. There is a possibility that during the study, you may experience worsening of any side effects you currently

have with pegvisomant, or may develop new side effects. Potential side effects from pegvisomant use include diarrhoea or constipation, nausea, headaches and dizziness. It may also affect your glucose levels and if you are diabetic you may require less diabetes medication; we will regularly review this during the study. More severe side effects that have been reported are rare but include abnormal liver tests, although in all patients who have experienced this, liver tests have returned to normal once the pegvisomant has stopped. We will therefore have to monitor your liver tests during the study.

Studies of pegvisomant have included some patients taking higher doses (more than 30mg per day) and there is no evidence that a higher dose increases the risk of side effects. If you do develop any symptoms such as fatigue whilst on the higher dose, please inform us straight away and you may need to withdraw from the study.

Also the purpose of the study is to slightly over-treat you with pegvisomant and so we will be intentionally lowering your IGF1 level to below the normal range. As this study only lasts for 12 weeks and as we are only aiming for IGF1 levels which are just below the normal range, we would not expect to cause any symptoms of growth hormone deficiency. Symptoms tend to only occur when growth hormone deficiency is severe and include tiredness and lack of motivation.

If you notice any new symptoms during the course of this study please inform your study doctor. If any symptoms or problems arise which are considered to be serious, this will be reported as per standard practice to the Medicines and Health Regulatory Agency.

We will also be performing DEXA scans to monitor your body composition at the start and at the end of the study. These involve a low level of radiation, equivalent to the level you would be exposed to during a 2 hour short haul flight.

What happens when the research study stops?

You will continue to receive your usual care at the endocrinology department at St Bartholomew's Hospital throughout the course of this study and after it concludes.

What happens to the blood samples?

The required tests will be performed within the biochemistry laboratory. After the completion of the study, the samples will be destroyed.

What happens to all data?

All data from this study will be stored in a file within a Barts and the London trust computer that will only be accessible by members of the research team.

If you join the study, some parts of your medical records and the data collected for the study may be looked at by representatives of regulatory authorities and by authorised people from Barts and the London Trust, other NHS bodies to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

All information which is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research the people conducting the study will abide by the Data Protection Act 1998, and the rights you have under this Act.

With your consent, we will inform your GP of your participation in this study but specific details regarding your results will not be disclosed without your consent.

What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, you will be told why and your continuing care will be arranged.

What happens if there is a problem?

We would not expect you to suffer any harm or injury because of your participation in it. If you are harmed by taking part in this study, there is no special compensation arrangement. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay your legal costs. Regardless of this, if you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Please contact Patient Advisory Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint. Please telephone 020 7377 6335, minicom 020 7943 1350, or email pals@bartsandthelondon.nhs.uk, you can also visit PALS by asking at any hospital reception.

Contact Details for further information

Further Information can be obtained from:

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Who is organising and funding the research and where was it reviewed?

This project has been organised by Dr WM Drake with Pfizer and has been peer reviewed. This study was given a favourable ethical opinion for conduct in the NHS by the East London and City REC.

Queen Mary University of London

Barts and The London NHS Trust

CONSENT FORM (Version 1 Dated 15/6/2007)

Title of project: A Study To Determine The Optimum Serum IGF-I Range In Patients With Acromegaly Treated With Pegvisomant.

Investigator: Dr WM Drake, Dr VJ Moyes

Centre Number: Patient Identification Number for this trial:

Please initial box to indicate agreement

1.	I confirm that I have read and understand the information sheet dated 04/10/07 (version 7) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities or from the Barts and the London/ Queen Mary University of London, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4.	I agree to my GP being informed of my participation in the study.	
5.	I agree to take part in the above study.	

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name (if different from Investigator)	Date	Signature
_____	_____	_____
Investigator	Date	Signature

1 copy for Patient, 1 for Investigator and original to be kept in medical notes

AcroQoL Questionnaire

Scale 1 (Physical, 8 items)	
Item 1	My legs feel weak
Item 3	I get depressed
Item 9	I have problems carrying out my usual activities
Item 13	The illness affects my performance at work or in my usual tasks
Item 14	My joints ache
Item 15	I am usually tired
Item 19	I feel like a sick person
Item 22	I feel weak
Scale 2 (Psychological, 14 items)	
subscale 2–1 (Appearance, 7 items)	
Item 2	I feel ugly
Item 4	I look awful in photographs
Item 7	I look different in the mirror
Item 11	Some parts of my body (nose, feet, hands,…) are too big
Item 12	I have problems doing things with my hands, e.g. sewing or handling tools
Item 16	I snore at night
Item 17	It is hard for me to articulate words due to the size of my tongue
subscale 2–2 (Personal relationships, 7 items)	
Item 5	I avoid going out very much with friends because of my appearance
Item 6	I try to avoid socializing
Item 8	I feel rejected by people because of my illness
Item 10	People stare at me because of my appearance
Item 18	I have problems with sexual relationships
Item 20	The physical changes produced by my illness govern my life
Item 21	I have little sexual appetite

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ORIGINAL ARTICLE

d3-GHR genotype does not explain heterogeneity in GH responsiveness in hypopituitary adults

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Summary

Objective Heterogeneity in growth hormone (GH) responsiveness in adult hypopituitary patients receiving recombinant human GH (rhGH) is poorly understood; doses vary up to fourfold between individuals. Deletion of exon 3 in the GH receptor (d3-GHR) has been linked to enhanced rhGH responsiveness in children. We investigated the role of the d3-GHR polymorphism in determining adult rhGH responsiveness.

Methods One hundred and ninety-four patients treated with an identical rhGH dosing protocol in a single centre were genotyped for the d3-GHR, and the results correlated with changes in serum IGF-I and clinical parameters of GH responsiveness after 6 and 12 months of GH replacement therapy.

Results Allele frequencies for homozygous full length (fl/fl), heterozygous d3 (fl/d3) and homozygous d3 (d3/d3) were 52%, 38·7% and 9·3%, respectively, and were in Hardy–Weinberg equilibrium. Baseline IGF-I and ΔIGF-I at 6 months were comparable between groups. ΔIGF-I at 12 months was significantly greater in the d3/d3 group ($P = 0{\cdot}028$). No difference was detected between fl/d3 and fl/fl groups. Regression analyses of ΔIGF-I at 12 months and ΔIGF-I/rhGH dose confirmed a significant relationship of d3/d3 genotype on rhGH response. There was no difference between groups in maintenance rhGH dose between genotypes.

Conclusion Homozygosity for d3-GHR confers a marginal increase in GH responsiveness at 12 months but without a detectable change in maintenance rhGH dose required. Both d3 alleles are required to achieve this response; given that only 10% of the population are d3 homozygotes, the d3GHR does not explain the marked heterogeneity of GH responsiveness in hypopituitary adults.

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Introduction

Since the original double-blind, placebo-controlled studies of recombinant human growth hormone (rhGH) treatment for adult growth hormone deficiency (GHD),[1–5] it has become apparent that doses of rhGH need to be individually tailored to patients to maximize clinical benefit and minimize the possible risks of prolonged excess GH exposure.[6] Most physicians experienced in the management of hypopituitary patients employ a strategy of dose titration, with monitoring of serum insulin-like growth factor-I (IGF-I), a GH-dependent peptide, as the main marker of excess dosing. Using such protocols, it is apparent that individual requirements for rhGH vary considerably between patients. For example, in a report of 50 consecutive patients treated with an identical dosing protocol that aims to maintain serum IGF-I between the median and upper end of the age-related reference range, median dose requirements for males and females, respectively, were 0·8 IU (range 0·4–1·6) and 1·2 IU (0·8–2).[7] Oestrogen is known to attenuate IGF-I production and may partially explain the variability between genders, but this does not explain why dose requirements vary fourfold and 2·5-fold in male and female groups, respectively.[7]

The factors responsible for determining individual GH responsiveness are currently unknown, but an obvious candidate is the GH receptor (GHR). The GHR gene is located on the short arm of chromosome 5 (p13.1-p12) and contains nine coding exons, with exons 3–7 encoding the extracellular ligand-binding domain.[8,9] A genetic polymorphism exists in the GHR resulting in the deletion of exon 3 and loss of amino acid residues 7–28; the overall prevalence is 25–32% with a homozygosity rate of 9–14%.[10,11] The effects of the loss of these amino acids are unknown; modelling of the residues by crystallography has been unsuccessful, but it appears not to influence the binding of GH to the GHR *in vitro*.[12,13] It has been speculated that this region may play a role in the conformational changes during activation of the GHR dimer by GH.[11]

The presence of one or more d3-GHR alleles has been shown to accelerate linear growth in children short for gestational age (SGA) or with idiopathic short stature (ISS) treated with rhGH,[11] although subsequent data have been conflicting.[14–17] In adults with GHD, similarly conflicting results exist. In one study of 99 patients, a greater IGF-I response was demonstrated in subjects carrying at least one d3 allele after 1 but not 5 years of rhGH treatment.[18]

However, in a separate recent report, no difference in rhGH responsiveness was demonstrated between d3+ or – genotypes in 124 patients treated for 1 year.[19] The reasons for these discordant results in adults and children are not clear, although one possibility is that by combining d3 homo and heterozygous groups for analysis, the full effect of the d3-GHR may be wholly or partially concealed. The aim of this study was to investigate whether, in the clinical setting, the d3-GHR polymorphism is an important contributor to variable GH responsiveness in adult hypopituitary patients treated with an identical dosing regimen of rhGH. In particular, we were interested to investigate differences in GH responsiveness between d3-GHR homozygotes and heterozygotes as this has not been explored in previous studies.

Patients and methods

This study was approved by the local ethics committee, and written informed consent was obtained from all patients. One hundred and ninety-four adult patients with GHD from a single centre were studied. All were treated with rhGH according to an identical dose titration protocol, previously described,[7] the stated aim of which was to maintain a serum IGF-I between the median and the upper end of the age-related reference range. The diagnosis of GHD was confirmed by a peak serum GH concentration ≤ 9 mU/l (3 mcg/l) during insulin-induced hypoglycaemia or on a glucagon stimulation test.

Details of the cohort are shown in Table 1. No patients had any identifiable reasons for altered IGF-I production, e.g. anorexia nervosa, renal failure, liver failure or concomitant use of opiates, DHEA supplements or levodopa. None had active Cushing's syndrome at the commencement of GH; those patients with a diagnosis of Cushing's were 3–22 years post cure/successful control of cortisol excess.

Following initial titration of rhGH dose, standard clinical and biochemical assessments were performed at 6 and 12 months and annually thereafter. For the purposes of this study, 6-month and 12-month clinical data, waist:hip ratio, fasting lipid profile and change in quality of life (AGHDA questionnaire), biochemical response to rhGH treatment (ΔIGF-I SDS) and maintenance dose of rhGH required were all analysed with respect to GHR genotype.

Table 1. Clinical features and demographics of the cohort studied

			n = 194	
			N	%
Gender	Male		81	41.8
	Female	Total	113	58.2
		Oestrogen replete	88	45.3
		Oestrogen deficient	25	12.8
Age at Diagnosis		<20	10	5.1
		21–30	15	7.7
		31–40	41	21.1
		41–50	56	28.8
		51–60	42	21.6
		>60	30	15.5
Pituitary Hormone Deficiency	Isolated Growth Hormone Deficiency		20	10.3
	Multiple Pituitary Hormone Deficiency		174	89.6
Cause of GHD	Pituitary tumour/treatment	Total	137	70.6
		NFPA	58	29.8
		PRL	34	17.5
		GH	9	4.6
		ACTH	36	18.5
	Pituitary apoplexy		9	4.5
	Inflammatory/Infective		9	4.5
	Trauma		3	1.5
	Congenital		2	1.0
	Other CNS tumours		25	12.9
	Other		8	4.1
Previous Radiotherapy	Yes	Total	124	63.9
		EBRT	122	62.9
		Gamma Knife Radiosurgery	5	2.6
	No		69	35.6
Duration of rhGH Treatment	Mean (months)		91	
	Range (months)		12–276	

GHD, growth hormone deficiency; E2, oestrogen; NFPA, nonfunctioning pituitary adenoma; PRL, prolactinoma; GH, Acromegaly; ACTH, Pituitary-dependent Cushing's syndrome; CNS, central nervous system tumours; EBRT, external beam radiotherapy; rhGH, recombinant human growth hormone.

To remove the potential confounding effect of rhGH dose, analyses were repeated using the ratio of IGF-I change to rhGH dose (delta IGF-I/rhGH dose) at 6 and 12 months.

Assays

IGF-I Assay: Between 1997 and 2005, an RIA kit (Diagnostic System laboratories Inc, Webster, TX, USA) with mean intra-assay and interassay coefficients of variation (CVs) of 7·2% and 10·4%, respectively, was used. From 2005 to present, serum IGF-I was measured by an automated solid-phase, enzyme-labelled chemiluminescent immunometric assay (Siemens Medical Solutions Diagnostics, Gwynedd, Wales, UK), with intra-assay and interassay CVs of less than 11% and 8%, respectively. For each individual patient, baseline, 6-month and 12-month IGF-I measurements were performed using the same assay (Table 4). To take into account, the change in assay during the time in which these patients were assessed and treated, data in this study have been analysed using standard deviation scores (SDS), calculated as *n*-mean/SD using age and gender related normative data obtained from our laboratory for each respective assay.

Serum GH was quantitated by an immunoradiometric assay using Immulite 2000 (Siemen's Medical Solutions Diagnostics), with interassay and intra-assay CVs of 5%.

Molecular studies

Genomic DNA was extracted from peripheral blood leucocytes using standard methods (BACC2 DNA extraction kit; GE Healthcare, Little Chalfont, Buckinghamshire, UK). A simple multiplex PCR was performed using three primers; forward TGTGCTGGTCTGTTGGTCTG, reverse full length GGATGCTA TGTCAGAGTCAG and reverse d3 GGTAAGTCACATAGA-TACTG (Sigma–Aldrich, Gillingham, Dorset, UK). A standard 45-cycle amplification of the GHR-exon 3 region was performed. Amplification products were analysed using 2% agarose gel stained with ethidium bromide. The fl allele is represented by a 521 bp fragment and the d3 allele by a 470 bp fragment (Fig. 1).

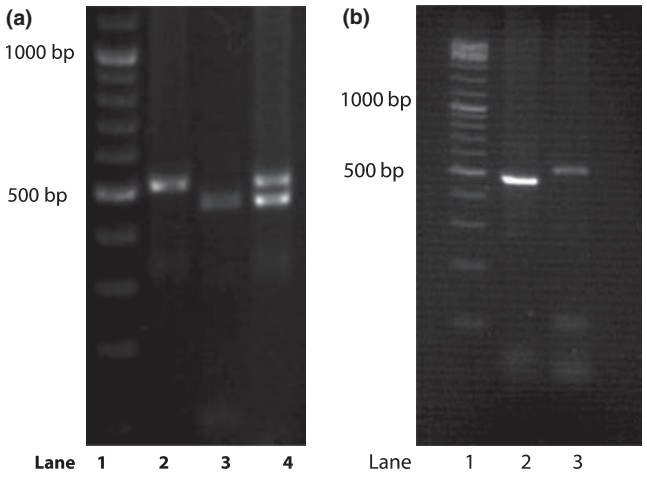

Fig. 1 Amplification products of multiplex PCR shown on agarose gel stained with ethidium bromide. The fl allele is represented by a 521 bp fragment and the d3 allele by a 470 bp fragment. (a) Lane 1 represents a genomic DNA ladder; lane 2 represents fl/fl homozygote; lane 3 represents d3/d3 homozygote and lane 4 represents fl/d3 heterozygote. (b) Comparison of PCR results for the same patient. Lane 1 is a genomic DNA ladder; lane 2 represents the results of 3 primer PCR demonstrating a single 470 bp band (d3/d3); lane 3 represents the same patient's result from a repeat PCR using only forward and full length reverse primers with a 521 bp band is visible, thereby confirming d3 heterozygosity.

Previous studies have suggested an over-estimation of the frequency of d3/d3 homozygotes presumably attributed to the fact that smaller PCR products are preferentially formed in a competitive reaction.[15] To exclude heterozygosity in the d3/d3 homozygotes, a second PCR was performed with only forward and full length reverse primers.

Statistics

Results are reported as mean value ± standard deviation (SD). Statistical significance was accepted at a P value <0·05. One-way ANOVA with Fisher LSD *post hoc* analysis was performed to compare rhGH response at 6 and 12 months, and the doses of rhGH required between the 3 genotype groups: fl/fl, fl/d3 and d3/d3. Comparison was also made of the change in biological parameters of GH activity; waist:hip ratio, quality of life (AGHDA) score and fasting lipid profile.

A stepwise multiple regression model was created to assess the individual and cumulative effect of genotype on GH response in conjunction with other potentially contributory factors: GH dose, oestrogen and external pituitary irradiation. To exclude the confounding effect of GH dose, analyses (ANOVA and multiple regression analysis) were performed using the ratio of delta IGF-I/GH dose at 6 and 12 months. Analysis was performed using SPSS (version 11.01; SPSS Inc, Chicago, IL, USA) for Windows XP (Microsoft Corp, Portland, Oregon, USA).

Results

Genotyping

The frequencies of the 3 genotypes (fl/fl, fl/d3 and d3/d3) were 52%, 38·7% and 9·3%, respectively, and were in Hardy–Weinberg equilibrium. Repeat PCR of d3/d3 patients using forward and full length reverse primers resulted in reclassification of 2/20 (9%) patients as heterozygotes (Fig. 1). Proportions of male and female subjects were comparable between genotype groups.

Serum IGF-I concentrations

The vast majority of patients maintained serum IGF-I levels within the 'target range' as previously reported. A very small number of patients had serum IGF-I levels outside of the reference range at the time of either 6 or 12 months (see SDS results in Table 2). Appropriate adjustments of rhGH dose were made at the time in clinical practice. Baseline IGF-I levels were comparable between all 3 genotype groups (Table 2).

Table 2. Comparison of results for the 3 GHR genotype groups using ANOVA with Fisher LSD *post hoc* statistical analyses. Response to treatment is demonstrated by ΔIGF-I and are reported as standard deviation scores (SDS). Further analyses of the ratio of IGF-I change to rhGH dose are included to exclude the potential confounding effect of variability in rhGH dose. Data are reported as mean and (standard deviation)

	fl/fl $n = 101$	fl/d3 $n = 75$	d3/d3 $n = 18$	ANOVA *P* values		
Gender						
Male	43	32	6	fl/fl *vs*. fl/d3	fl/fl *vs*. d3/d3	fl/d3 *vs*. d3/d3
Female	58	43	12			
IGF-I SDS						
Basal	−1·03 (1·36)	−1·14 (1·25)	−1·17 (1·19)	0·610	0·675	0·910
6 months	0·82 (1·68)	0·78 (1·60)	0·52 (1·55)	0·883	0·478	0·545
12 months	0·96 (1·49)	1·01 (1·61)	1·76 (1·52)	0·843	**0**·046	0·066
rhGH Dose (mg)						
6 months	0·37 (0·17)	0·38 (0·16)	0·37 (0·13)	*0*·802	*0*·953	0·933
Range	0·1–1·0	0·1–0·9	0·1–0·7			
12 months	0·41 (0·22)	0·41 (0·23)	0·43 (0·33)	*0*·906	*0*·701	0·658
Range	0·1–1·2	0·1–1·3	0·1–1·5			
ΔIGF-I SDS						
6 months	1·85 (1·88)	1·91 (1·49)	1·69 (1·63)	0·805	0·721	0·623
12 months	1·99 (1·49)	2·15 (1·74)	2·93 (1·29)	0·556	**0**·028	0·070
ΔIGF-I/rhGH Dose						
6 months	4·98 (11·1)	5·89 (6·89)	5·20 (5·03)	0·784	0·786	0·671
12 months	5·80 (5·58)	5·93 (5·65)	10·2 (8·68)	0·882	**0**·004	**0**·007
ΔAGHDA						
12 months	10·3 (6·66)	9·35 (6·00)	11·3 (5·75)	0·533	0·473	0·296
ΔWaist: Hip Ratio						
12 months	0·02 (0·16)	0·57 (0·16)	0·03 (0·08)	0·116	0·749	0·547
ΔChol mmol/l						
12 months	0·42 (1·26)	0·605 (1·17)	0·64 (1·44)	0·124	0·290	0·901
ΔTrig mmol/l						
12 months	0·18 (1·13)	0·14 (0·95)	0·38 (0·86)	0·993	0·267	0·279

fl/fl, full length homozygotes; fl/d3, d3 heterozygotes; d3/d3, d3 homozygotes; rhGH, recombinant growth hormone; AGHDA, QOL score; Chol, cholesterol; Trig, triglycerides.

rhGH response at 6 months

Comparison of the IGF-I response to rhGH at 6 months demonstrated no significant difference between the 3 genotype groups, and the doses used were equivalent (Table 2, Fig. 2a). A multiple linear regression model did not detect any individual or cumulative relationship between genotype, rhGH dose, oestrogen or external pituitary irradiation and ΔIGF-I SDS (Table 3). Comparison of the ratio of ΔIGF-I to rhGH dose also demonstrated no significant difference between genotype groups at 6 or 12 months (Tables 2 and 3).

rhGH response at 12 months

A significant difference between the fl/fl and d3/d3 genotype groups was detected for ΔIGF-I achieved at 12 months using

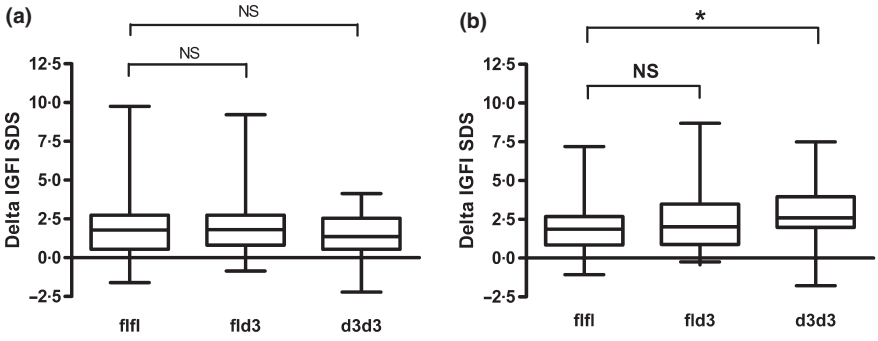

Fig. 2 Box plots to demonstrate the variability in ΔIGF-I response between GHR genotype groups. *denotes statistical significance ($P < 0·05$) between fl/fl and d3/d3 groups using ANOVA comparing ΔIGF-I at 12 months. NS denoted no statistical significance. (a) ΔIGF-I between baseline and 6 months. (b) ΔIGF-I between baseline and 12 months.

Table 3. Results of multiple regression analyses investigating factors influencing GH responsiveness compared to full length homozygotes (fl/fl). Further analyses of the effect of genotype on the ratio of IGF-I change to rhGH dose were performed assessing the change per unit dose. To confirm the relationship between oestradiol and GH responsiveness, a stepwise regression analysis was performed including and excluding pituitary irradiation (RT); results confirmed a lack of a statistical significance

	Baseline–6 months				Baseline–12 months			
	ΔIGF-I		ΔIGF-I/Dose		ΔIGF-I		ΔIGF-I/Dose	
	β-Coeff	*P* value	β-Coeff	*P* value	β-Coeff	*P* value	β-Coeff	*P* value
fl/d3	0·019	0·799	0·022	0·770	0·053	0·477	0·006	0·940
d3/d3	−0·023	0·755	−0·016	0·835	0·161	**0**·029	0·214	**0**·004
Oestrogen (excluding RT)	**0**·088	**0**·432 (0·473)	**−0**·094	**0**·197 (0·136)	**0**·06	**0**·422 (0·171)	**−0**·122	**0**·089 (0·072)
Pituitary Irradiation	0·094	0·231	0·107	0·144	0·046	0·528	0·043	0·549
GH Dose	−0·059	0·203	N/A	N/A	0·142	0·059	N/A	N/A
R Squared	**0**·020		**0**·024		**0**·054		**0**·061	

Table 4. This table shows delta IGF-I results (ng/ml) for each genotype for each assay, pre-2005 and post-2005. Cumulatively, we report the percentage change in IGF-I achieved between baseline and 6 and 12 months

	ΔIGF-I (ng/ml)							
	Assay 1 (pre-2005)			Assay 2 (post-2005)				
	N	0–6 months	0–12 months	N	0–6 months	0–12 months	% Change IGF-I 0–6 months	% Change IGF-I 0–12 months
fl/fl	79	88·8 (71·0)	82·9 (67·5)	22	82·8 (43·3)	89·9 (65·1)	101·3	101·7
fl/d3	59	106·0 (54·0)	108 (80·0)	16	79·0 (44·1)	93·0 (63·4)	123·4	120·4
d3/d3	15	85·7 (81·4)	110·3 (69·2)	3	100·0 (30·8)	83·0 (25·9)	124·7	138·5

ANOVA ($P = 0{\cdot}028$). There was no difference detected between the fl/fl and fl/d3 group ($P = 0{\cdot}556$) or between the fl/d3 and d3/d3 groups ($P = 0{\cdot}07$). The maintenance dose of rhGH required to achieve target IGF-I was also comparable between genotype groups (Table 2). Analysis of the ratio of IGF-I change to rhGH dose confirmed the significant difference in the d3/d3 group ($P = 0{\cdot}004$ compared to fl/fl and 0·007 compared to fl/d3) using ANOVA.

Multiple linear regression analysis confirmed the positive relationship between d3/d3 genotype and ΔIGF-I between baseline and 12 months ($P = 0{\cdot}029$) compared to fl/fl genotype. There was no significant relationship for fl/d3 genotype or rhGH dose. Repeat multiple regression analyses assessing the ratio of IGF-I change to rhGH dose confirm the significant effect of d3/d3 genotype ($P = 0{\cdot}004$) (Table 3).

Effect of oestrogen and pituitary irradiation

The influence of oestrogen and pituitary irradiation on long-term rhGH response was also investigated as part of the multiple regression models; no significant relationship was detected for either variable at 6 or 12 months. Regression analyses were performed in a stepwise manner with nonsignificance demonstrated for oestradiol with and without the inclusion of pituitary irradiation (for delta IGF-I at 12 months, $P = 0{\cdot}171$ without inclusion of pituitary irradiation and 0·422 including pituitary irradiation in the analyses. For delta IGF-I/rhGH dose, $P = 0{\cdot}072$ without inclusion of pituitary irradiation and 0·089 including pituitary irradiation in the analyses).

The vast majority of female hypogonadal patients were taking oral rather than trans-dermal oestrogen; statistical power was insufficient for subgroup analysis.

Biological markers of GH activity

Baseline biological markers were comparable between groups (Table 2). Analysis of the change in AGHDA score, waist: hip ratio and fasting lipid profile did not demonstrate any significant difference between genotype groups.

Discussion

To our knowledge, this is the largest study to date of the effect of the d3-GHR genotype on GH responsiveness in hypopituitary patients treated with GH. By studying 194 patients, all treated with an identical dose titration regimen, we have been able to perform an extensive statistical analysis, including an assessment of the effects of d3 homo and heterozygosity; previous studies, both in acromegaly and hypopituitarism,[18,20,21] have grouped these patients together. The data suggest that homozygosity for the d3-GHR may confer a marginal increase in GH responsiveness at

12 months, as demonstrated by an augmented IGF-I response from baseline over this time period. There was no difference in the doses of rhGH between groups and analyses of the ratio of change of IGF-I to rhGH dose confirmed this significant effect, thereby suggesting that a greater change in IGF-I was achieved for a given rhGH dose in d3 homozygous subjects. These data are in keeping with a previous report,[18] although the smaller size of that study dictated that d3 homozygotes and heterozygotes were analysed together. Shorter-term GH responsiveness (ΔIGF-I between baseline and 6 months, which includes the period of dose titration) was comparable between groups; the reason(s) for the discrepancy between responses at 6 and 12 months is not clear.

Given the enhanced GH responsiveness evident in the d3 homozygous patients at 12 months, one might have expected this to translate into a lower maintenance dose requirement for GH to achieve an equivalent 'target' serum IGF-I. However, no statistically significant differences in rhGH dose requirements were evident between genotype groups. One possible explanation for this is the small number of d3 homozygotes: 20 patients, despite the large scale of our study. A second, probable, contributory reason is the design of the GH dose titration regimen used.[7] Patients commenced 0·8 IU (more recently, 0·3 mg) daily, with regular measurement of serum IGF-I and dose adjustments as required, aiming to maintain a value between the median and upper end of the age-adjusted normal range. For the vast majority of male patients, this starting dose resulted in a serum IGF-I within the 'target range', with no requirement for subsequent dose increments. The 'target range' for this dose titration protocol is wide, for example, the normal range for a patient aged 21–30 is 117–358 ng/ml, with a median of 176, thereby giving a 'target range' of 182 ng/ml. Although no increments in dose were needed for the majority of patients above 0·8 or 1·2 IU for males and females, respectively, statistically significant differences in IGF-I values at 12 months were noted between genotype groups, but *within* the target range. This would suggest that, although heterogeneity in GH responsiveness between genotype groups is evident at 12 months, this is not of particular clinical relevance and does not explain the marked variation in rhGH doses observed in clinical practice.

Previous studies of the effect of the d3-GHR on GH responsiveness have largely been confined to children. Data have been conflicting with enhanced linear growth responses to injected GH observed in some, but not all, patients with ISS, SGA, GHD and Turner syndrome.[11,14,17,22–24] Two recent studies of adult GHD patients have shown similarly conflicting results. Enhanced IGF-generation was demonstrated after 1 but not 5 years therapy with GH in d3-GHR homozygotes and heterozygotes, in conjunction with changes in fasting lipid profile.[18] However, a more recent study has demonstrated no differences in IGF-I response or reductions in body fat between d3 genotype groups after 1 year of rhGH treatment.[19] Conflicting data regarding IGF-I levels in response to a GH stimulus have also been shown in adult acromegalic patients both preoperatively and postoperatively.[20,21] There are two possible reasons for the lack of concordance between studies. First, it is possible that there is variability in the response of d3 homozygotes and heterozygotes; we postulate that this may be because of functional differences in the GHR formed. Assuming equivalent affinity of receptor dimers, only 25% of the GHRs of heterozygous patients will be d3/d3 homodimers. If a d3/d3 homodimer is required for augmented GH responsiveness, then a d3 heterozygote will exhibit only 25% of the 'extra' IGF-I generation capacity, an effect that may not be detected in smaller studies. More detailed studies of the transcriptional activity of hetero *vs*. homodimers are needed to determine whether there is a difference in functional activity and thus whether there is a partial increase in GHR response in d3 heterozygotes in comparison with d3 homozygotes. Second, repetition of the PCR with two primers has ensured that our classification of subjects as d3 homozygotes or heterozygotes is robust; previous studies have shown that 20% of d3 heterozygotes are misclassified as homozygotes without this additional step.[15,16,25] As the majority of previous studies have not performed this second PCR, it is possible that a significant proportion of subjects have been mislabelled. If GH responsiveness varies between d3 homozygotes and heterozygotes, such genotype misclassifications may have contributed to the inconsistency of published clinical data.

In conclusion, this large-scale extensive study of the effect of the d3-GHR genotype on rhGH responsiveness in adult hypopituitary patients has demonstrated an increase in rhGH responsiveness at 12 months. However, given only 10% of the population are homozygous for d3-GHR and the marginal nature of the increased response, this is unlikely to explain the marked variability in rhGH dose requirements in this patient group, and further studies are needed before more tailored approaches to rhGH dosing can be developed.

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